AGEING IN DOGS (*CANIS FAMILIARIS*) AND ITS RELATIONSHIP TO THEIR ENVIRONMENT

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Table of contents

List of tables

List of figures

Abbreviations

ACTH- Adrenocorticotropic hormone, also adrenocorticotropin or corticotropin

ACTH- Corticotropin-releasing hormone

DNA- Acid deoxyribonucleic

HPA - hypothalamic–pituitary–adrenal axis

PCR- Polymerase chain reaction

POT1- Protection of telomeres protein1

qPCR- Quantitative polymerase chain reaction

RAP1- Repressor/Activator protein 1

rTL – Relative Telomere Length

TIN 2- TRF1 interacting protein 2

TRF- Telomeric restriction fragment

TRF1- Telomeric repeat binding factor 1

TRF2- Telomeric repeat binding factor 2

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Abstract

Animal welfare is assumed to be influenced by the cumulative effects of positive and negative events experienced by an individual. The present study investigated the levels of cortisol, relative telomere length and the association of these variables with the perception of canine age (apparent age). Firstly, it was investigated whether dogs' telomere lengths from dried blood correlated with the ones measured from oral swabs from the same individuals. As a correlation between samples from these two tissues were found we validated the use of buccal swab sampling to obtain dogs' relative telomere length. We then investigated whether dogs' relative telomere length present in buccal cells co-varies with glucocorticoid levels, results showed association between these factors. Since glucorticoids are the most used physiological parameter to assess animal welfare our results suggest the use of relative telomere length as a potential to evaluate animal welfare. We investigated if the different backgrounds of dogs were associated with relative telomere length. Results showed that dogs from different background, sex and with different activity levels had significantly different relative telomere lengths. We investigated whether dogs' relative telomere length would vary over a one-year period, and results showed that the implementation of social enrichment can increase relative telomere length of laboratory dogs. Finally, we investigated if a novel approach, estimating apparent age, could be used as a tool to assess welfare. Results showed that a dog's apparent age assessed from photographs could, potentially, be used as an animal welfare assessment method since people were able to identify dogs that were prematurely ageing. In conclusion, telomere attrition and apparent age can be used to indicate a dog's welfare.

Chapter 1 General Introduction

1.1 Animal Welfare Assessment

Humans have surrounded themselves with animals since the Neolithic revolution, either to stabilize their food resources, for guarding or helping in hunting, the fact is domestication has played a major role in the formation of human societies (Driscoll, Macdonald, & O'Brien, 2009) . Because of this close contact with domestic species, humans started to develop the concept of animal welfare as moral systems evolved, especially in modern times (Broom, 2011). The confinement of farm animals was one of the first topics to be instigated in the animal welfare debate (Hemsworth, Mellor, Cronin, & Tilbrook, 2015a). The notion of animal welfare at this time came mainly from farmers and veterinary surgeons; it was mostly associated with the animals' bodily integrity (i.e. if they were being fed, offered shelter, etc.) and whether they were producing, as a sign of positive welfare (Hewson, 2003).

The human concepts of moral actions, what is acceptable and what is not, have significantly changed over time, a better understanding of animals cognition, motivation and the complexity of social behaviour has resulted in the fast development of animal welfare science over the past few decades (Fraser, 2008b). Scientists have proposed different concepts and definitions of animal welfare, which have resulted in divergent methodologies and ways of understanding experimental data (Fraser, Weary, Pajor, & Milligan, 1997). The most common definition used is that the wellbeing of animals is related to how they cope with their environment and it can be said that the welfare of an animal is undoubtedly affected by both failure to cope and difficulty in coping (Broom, 1991).

Scientists have different perspectives regarding ways to assess animal welfare, some will emphasise health, physiological functioning and absence of diseases, while others will consider freedom to express natural behaviour, whereas others consider animals affective states, through positive or negative experiences, such as fear (Fraser, 2008a). The two main parameters used for welfare evaluation are behaviour and physiology, where the first one considers if the animal is allowed to perform species natural behaviour, including behaviours such as playing, courtship and copulation, foraging or if the animal performs abnormal behaviours. The second parameter investigates the animal's biological responses underlying attempts to cope, such as changes in body temperature, heart rate, cortisol (stress hormone) levels and immunological functioning (Hemsworth, Mellor, Cronin, & Tilbrook, 2015b; Hill & Broom, 2009; Passantino, Quartarone, Pediliggeri, Rizzo, & Piccione, 2014).

The discussion that once arose from farm animals currently includes the welfare of laboratory animals, shelter animals, working animals and zoo animals (Bassett & Buchanan-Smith, 2007; Cafazzo et al., 2014; Hart, Zasloff, Bryson, & Christensen, 2000; Hill & Broom, 2009; Overall & Dyer, 2013). For farm animals the issue moved from only focusing on better production to also consider the quality of life animals experience (Broom, 2011). Since the use of animals and the ethics around it are being intensively discussed, consumers are becoming more interested about the ethics behind the products they consume and expecting their products to be produced and processed with better respect for the animal's welfare (Biesalski et al., 2003; Broom, 2011; Seeker, Ilska, Psifidi, Wilbourn, Underwood, Fairlie, Holland, Froy, Salvo-Chirnside, et al., 2018; Waiblinger et al., 2006).

The same public awareness regarding animal welfare is observed for zoo animals, where the public is attentive to enclosure size, complexity and the affective

states of animals (Afonso, Berdasco, Medeiros, & Rejowski, 2012; Bassett & Buchanan-Smith, 2007; Fernandez, Tamborski, Pickens, & Timberlake, 2009; Hill & Broom, 2009). Pet owners are another segment of the population that are aware and concerned about their animal's quality of life and current considerations are a better diet, exercise and human-pet interaction (Marinelli, Adamelli, Normando, & Bono, 2007; Schneider, Lyons, Tetrick, & Accortt, 2010; Wojciechowska & Hewson, 2005).

Defining animal welfare can be challenging because of its complex nature, while the impact of some adverse stimuli are easy to detect such as food deprivation, injury, or poor housing others such as novelty or confinement could be more difficult to detect and measure (Broom, 1991; Protopopova, 2016). Physiology and behaviour are the main approaches used for measuring poor welfare, however, interpreting their outputs are not always straightforward (Mason & Mendl, 1993). In addition, procedures to obtain physiological or behavioural samples may offer different levels of difficulty depending on the size of enclosure or if the animal is extensively on intensively managed, for example (Turner & Dwyer, 2007). Evaluating the absence of abnormal behaviour can be time-consuming and requires the knowledge of the species natural behaviours and physiological measures will require laboratory analysis, which are not always practical and can be expensive (Cafazzo et al., 2014; Hill & Broom, 2009; Miller, Pisacane, & Vicino, 2016).

1.2 Evaluation of stress levels as a measure of animal welfare

Too much stress is one of the major problems related to animal welfare and understanding the mechanisms associated with animal welfare assessment is important for species' management since high levels of stress affect the individual's health (Van der Weyde, Martin, & Paris, 2016). The manner in which animals cope

with environmental challenges and change is highly related to the stress response (Sheriff, Dantzer, Delehanty, Palme, & Boonstra, 2011). Stress is a term that is generally used to describe the behavioural or physiological response of an animal to perceived threats or aversive stimuli (Hill & Broom, 2009). The hypothalamic-pituitary-adrenal axis (HPA) is responsible for initiating a cascade of hormonal and behavioural responses to a stressor, which redirects energy to the performance of survival or "emergency" behaviours (Owen, Swaisgood, Czekala, Steinman, & Lindburg, 2004). The response to a stressor is characterized by the release of corticotrophin-releasing hormone (CRH) via the hypothalamus. When this hormone (CRH) binds to its receptors the adrenocorticotropic hormone (ACTH) is released by pituitary gland. The ACTH binds to receptors on the adrenal cortex and stimulates adrenal release of glucocorticoids. Cortisol may be released for several hours after encountering the stressor (Möstl & Palme, 2002a). When a certain cortisol concentration is maintained in the blood it exerts negative feedback to the hypothalamic release of CRH and the pituitary release of ACTH (negative feedback) and then the systemic homeostasis returns when the stressor is moderate and the contact with it is brief (Tsigos & Chrousos, 2002) (*Figure 1*).

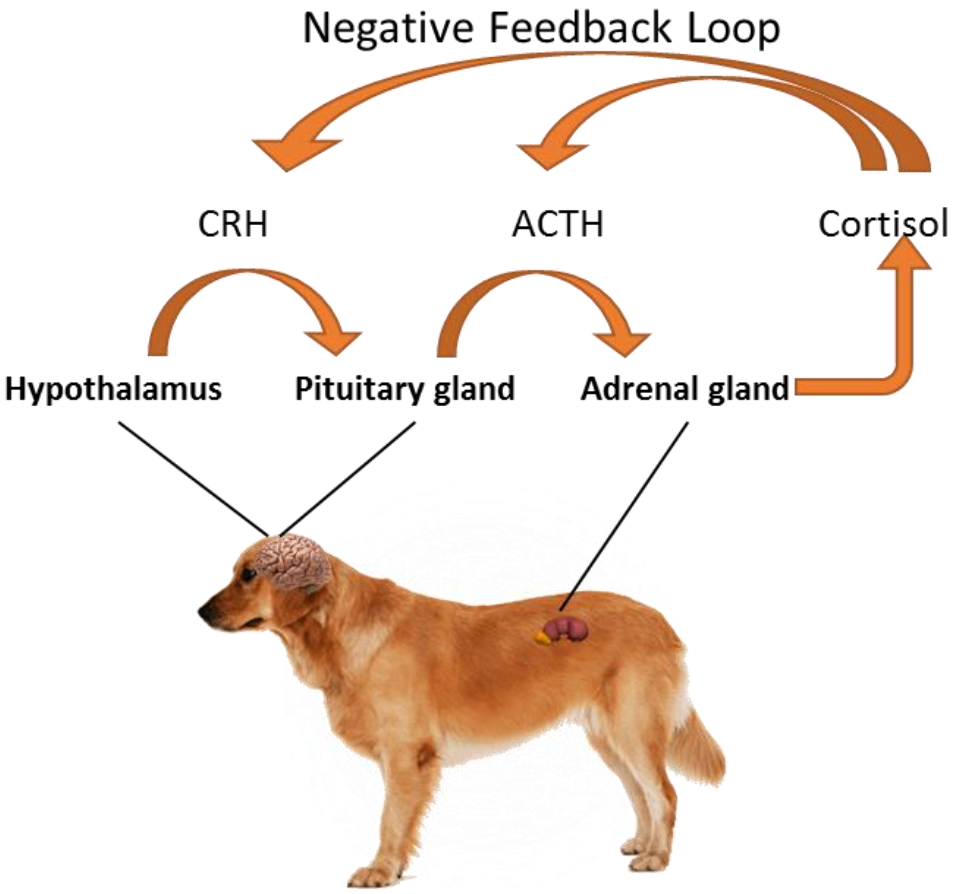

Figure 1: The response of the HPA-axis to a stressor adapted from Protopopova (2016).

The behavioural response, which is usually flight or fight is activated in part by adrenal gland glucocorticoid secretion (Bonne Beerda, Schilder, Van Hooff, De Vries, & Mol, 1998; Sapolsky, 2000). The steroid hormone secretion facilitates the mobilisation of glycogen stores that serve as a ready source of energy for the appropriate behavioural response (Owen et al., 2004).

When the stimulus is very intense or constant chronic stress is developed, which leads to elevated levels of HPA axis hormones; that is, glucocorticoids, ACTH and corticotrophin releasing hormone that may have deleterious effects on the individual. These effects includes inhibition of normal reproductive function, suppression of the immune system, and tissue atrophy, all consequences of the body not being able to return the systemic homeostasis (Young et al., 2004). Measuring glucocorti-

coid levels (or metabolites of this hormone) allows not only the evaluation of animal welfare, but also to define animals' management strategies (Teixeira, de Azevedo, Mendl, Cipreste, & Young, 2007). Stress responses can be triggered by a number of factors, such as exposure to novelty, lack of predictability or controllability of important events (Möstl & Palme, 2002a). Glucocorticoid concentration in the blood gives information about stress levels, however, the sample collection could itself be a stressor and often it is not feasible to blood sample wild species (Palme & Möstl, 1997). One of the techniques for noninvasively monitoring glucocorticoid levels in wild animals is the measurement of faecal glucocorticoid metabolites (Palme, 2005). One of the advantages of this method is that hormone metabolites can be collected without capturing and disturbing the animal (Hulsman et al., 2011). Currently, this method is used to investigate questions about hormone-behaviour interactions, reproduction, animal welfare and conservation (Van der Weyde et al., 2016). But the disadvantage is that it only provides a mean measurement of an animal's welfare over the period of the intestinal transit of each species, dogs for example takes 24 hours; that is, it cannot detect acute stressors. Furthermore, a baseline level needs to be established for each individual since it is the elevated presence of this hormone that indicates an animal welfare problem and not the mere presence of the hormone (Touma & Palme, 2005).

Over time elevated levels of cortisol, a glucocorticoid, are potentially harmful to tissues, and its associated as well with a greater prospect of the development of cardiovascular disease and suppressed immunity (Sapolsky, 2000). Whereas, basal levels of cortisol may be indicative of healthy ageing (Noordam et al., 2012).

1.3 Telomeres as biological clocks

Telomeres are structures of non-coding DNA-protein complexes at the ends of chromosomes and are composed of repeats of a six nucleotide unit sequence TTAGGG that extend for thousands of bases at chromosome caps (Aydinonat et al., 2014). Telomeric DNA is associated with a specialized multi protein complex, formed by six proteins: TRF1 (Telomeric repeat binding factor 1), TRF2 (Telomeric repeat binding factor 2), RAP1 (Repressor/activator protein 1), TIN2 (TRF1 interacting protein 2), TTP1 (TRF1-interacting nuclear protein 1) interacting protein) and POT1 (Protection of telomeres protein 1) (Armanios & Blackburn, 2012). This structure that is essential to shield the exposed ends of telomeres protecting the chromosome called she (Shay, 2018). In its structure telomeres have a displacement loop or D-loop, that is a DNA structure where the two strands of a double-stranded DNA molecule are separated for a stretch and held apart by a third strand of DNA; and it also has a T-loop, a loop similar to a knot, which stabilizes the telomere, preventing the telomere ends from being recognized as break points by the DNA repair machinery (Greider, 1999) (*Figure 2*). Telomere function is to protect the chromosome DNA coding regions from recombination, degradation, fusion, damage during cell division, they are therefore essential for the maintenance of genomic integrity (Gunn et al., 2008).

Research shows that telomeres play a role in cellular ageing because the DNA at the very end of the chromosome cannot be fully copied in each cell division, resulting in a slow, gradual shortening of the chromosome. When eventually reaches a short length it triggers senescence (Monaghan, Charmantier, Nussey, & Ricklefs, 2008) (*Figure 3*).

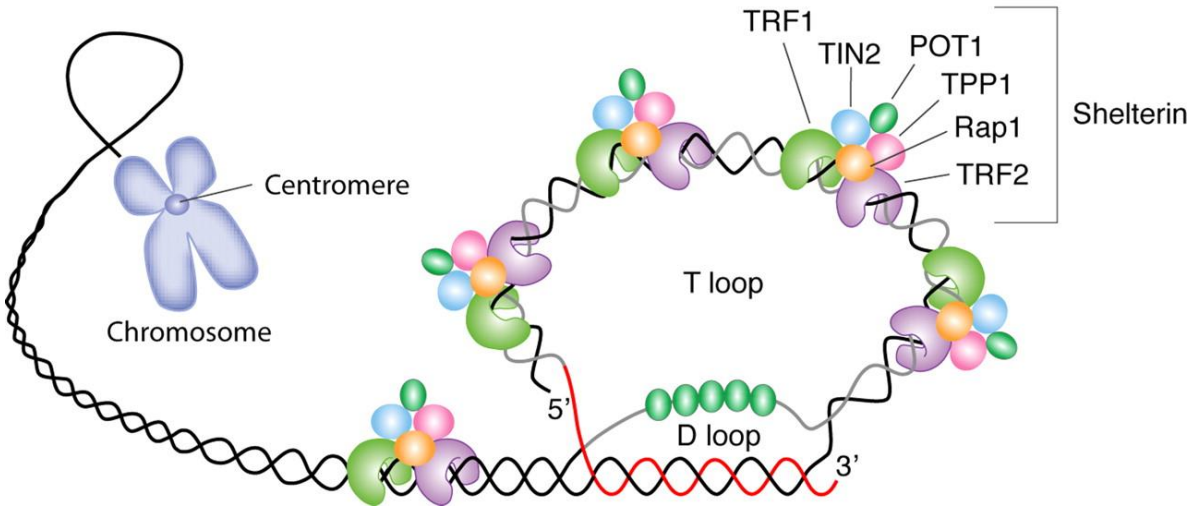

Figure 2 Telomere structure, D-loop and T-loop regions and Shelterin complex with its proteins. (Source: Calado & Young, 2008)

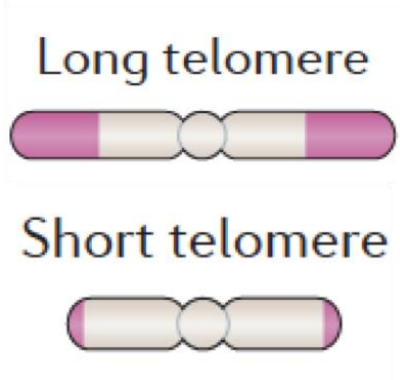

Figure 3 Telomere length comparison. (Source: Armanios & Blackburn (2012).

The replication of telomere-DNA involves telomerase, a specialized enzyme, which by copying a short template sequence from RNA can synthesize the telomeric DNA and therefore extend the chromosome's end (Blackburn, 2005) (*Figure 4*). Although telomeres can be repaired by the enzyme telomerase, this enzyme abundance varies between species, tissues and throughout development (Aydinonat et al., 2014). In germline cells telomerase provides a mechanism for the maintenance of telomere length as they proliferate indefinitely (Nasir, Devlin, Mckevitt, Rutteman, & Argyle, 2001). In somatic tissues, the telomerase is repressed immediately after birth, then its levels are insufficient to compensate the telomeric attrition during so-

matic tissue proliferation, consequently telomeres shorten with age from birth; thus, the length of the telomeres is a biological clock that effectively counts the cell divisions of the organism (Bateson, 2016).

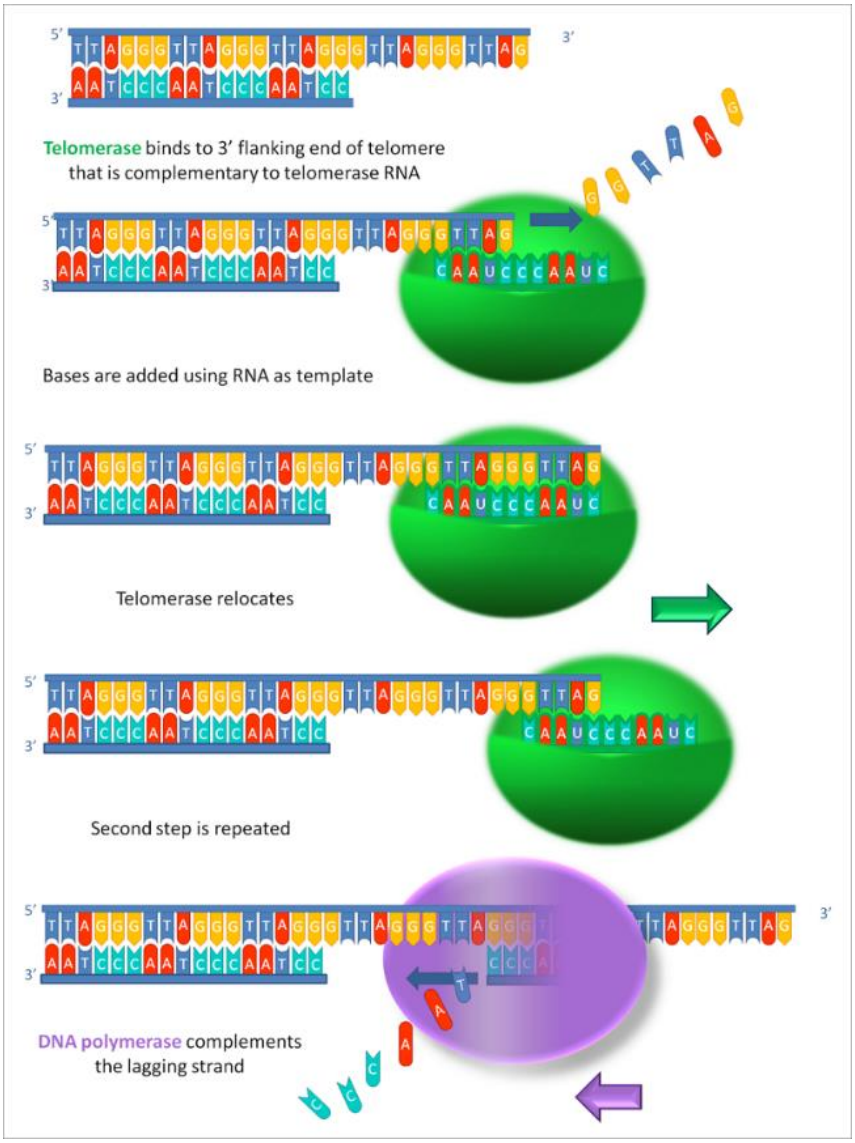

Figure 4 Telomerase repairing telomeric DNA. (Source: Wikiwand, on 20/03/19)

Telomere maintenance in humans is influenced by several factors such as psychological stress, diet, exercises and behavioural habits such smoking (Arsenis, You, Ogawa, Tinsley, & Zuo, 2015; Mirabello et al., 2009). Stressful events usually have a severe negative impact on telomere length (i.e. accelerate reduction) whereas, positive experiences - such as healthy diet, exercise and good sleep quality -

may delay, prevent or possibly reverse the effect of telomere attrition (Bateson, 2016). This is evidence that telomere length is an indicator for either positive or negative stimuli (welfare state).

Studies show that depression, posttraumatic stress disorder, being a refugee (i.e. displaced from their ancestral home), being exposed to pain, childhood traumas, financial stress, women suffering from intimate partner violence, are negative situations that exposure individuals to stress hormones, which are associated with the down-regulation of telomerase activity, the main enzyme responsible for the maintenance of telomeres (Cai et al., 2015; Choi, Fauce, & Effros, 2008; Küffer, O'Donovan, Burri, & Maercker, 2016; Zahran et al., 2015). Whereas having 33 minutes of vigorously physical activity training per week, a healthy diet, including greater intake of antioxidant, less processed meat consumption, intake of fruits and vegetables and less dietary fat, meditation training in a retreat setting are all associated with the reduction of cortisol and/or inflammation levels and as a result telomerase activity augmentation (Boccardi et al., 2013; Conklin et al., 2018; Puterman et al., 2010).

Since telomere conservation can be influenced by numerous factors rather than trying to determine the specificity of its maintenence it can be more valuable if contemplated as reflecting ageing (Blackburn, 2010). Ageing reduces the structural integrity and functional performance of tissue over time, the measurements of these changes are representative of the biological age of the tissues (Gunn et al., 2008). Once the measurement of biological age has been precisely established, it can be used to investigate the influence of environmental and genetic factors on telomere shortening and, therefore, it can be used to study the aetiology of ageing (Gunn et al., 2009). Possibly, in future assessing telomere length will be used as an integra-

tive indicator of health, as standard hemograms are used currently (Blackburn, 2010).

1.4 Apparent age as a measure of life experience

The speed of ageing varies between individuals in the same way that we grow at different rates. Since the 1980s the concept of age has been investigated and debated resulting in concepts such as: biological age – the age related to the body's physiology and cells; chronological age – refereeing to the actual amount of time that an individual has been alive (Borkan & Norris, 1980). In humans, 'apparent age' - usually the estimated chronological age of an individual - is a validated parameter for assessing patient health because it is associated with the patient's physiological state (Christensen et al., 2004).

The evaluation of apparent age, in humans, typically takes into consideration physical changes that happen with ageing progression, for example, alterations in facial skin such as presence wrinkles, sagginess, pigmentation alterations such as freckles and age spots (Porcheron, Mauger, & Russell, 2013). For assessing apparent age several studies use digital photographs (passport-type) where images were cropped at the neck up to the top of the head, and the subjects presented a neutral expression (Christensen et al., 2004; Gunn et al., 2013, 2009).

It has been found that individuals who look older than their chronological age had more health problems related to their apparent age than to their chronological age (Borkan & Norris, 1980; Gunn et al., 2008). A study highlighted that "looking older for your age" when identical twins were compared between each other was associated with increased mortality in a group of 387 twin pairs (Christensen et al., 2004). Researchers showed that apparent age was a feature that could predict familial lon-

gevity and cardiovascular diseases in a group of 670 individuals (Gunn et al., 2013). Therefore apparent age is a feature recommended to be used by medical doctors because in general it relates to an individual's health and for individuals older than 70 years old it predicts survival and associates with physiological and functional ageing (Christensen et al., 2009).

Human gerontology, the scientific study of the process of ageing, is a growing field of investigation, despite this the use of apparent age as a health indicator is not widely investigated yet. For non-human species studies are even more sparse, one study investigated the association between personality and health in facial appearance of chimpanzees (*Pan troglodytes*) and revealed that evaluators could infer about the apes' health status from photographs (Kramer, 2012). No similar study has been conducted for dogs, however, ageing studies already point out some important features related to dogs appearance that can be used when assessing dogs ageing health such as texture of skin, hyperpigmentation alopecia, cataracts, and greying hair (Bellows et al., 2015; King, Smith, Grandin, & Borchelt, 2016).

Several studies have associated chronic stress and apparent age where high levels of cortisol are associated with a higher perceived age, for example, people under financial stress tend to look six years older than their chronological age (Agrigoroaei, Lee-Attardo, & Lachman, 2017; Noordam et al., 2012). A study investigated the association between apparent age and leucocyte telomere length finding a significant correlation between the age assessed from photographs and the promising molecular biomarker of ageing (Christensen et al., 2009). No studies have been conducted with non-human mammals that associate chronic stress, perceived age and telomere length combined.

1.5 Canids as a model group

Canids were chosen to be our model group for the present study for the following reasons: 1) the domestic dog (*Canis familiaris*) is a species that is much studied and for this reason abundant data is available; 2) it is also a species with great availability of individuals, which is not true for wild species; 3) canids are good models for carnivores in general; and 4) domestic dogs are easy to sample from as most have obedience training. Furthermore, once this new assessment method has been validated, other species of carnivores in captivity may benefit from it.

For a number of reasons, the domestic dog is an interesting model to investigate different issues concerning animal welfare, evolution and cognition (Cooper et al., 2003; Rehn & Keeling, 2011; Trut, Plyusnina, & Oskina, 2004). Studies with dogs are carried out to understand wolves' domestication, to understand the communication between dogs and owners, and to understand cognition and learning (Boyko et al., 2009; Kaulfuß & Mills, 2008; Thorpe et al., 2006). Besides being companion animals, dogs are also used for work, such as patrol dogs, sniffer dogs, search and assistance dogs (Horváth, Igyártó, Magyar, & Miklósi, 2007; Jalongo, Astorino, & Bomboy, 2004). Currently, dog welfare issues are widely discussed because they reflect the growth of the pet market (Boya, Dotson, & Hyatt, 2012; Downes, Canty, & More, 2009). Therefore, results of studies with dogs, besides having animal welfare and scientific relevance will be of economic interest.

Dogs are housed under many different management routines: pets, police, shelters, laboratories, strays, etc which also makes them a good model (Downes et al., 2009; Hart et al., 2000; Luescher & Tyson Medlock, 2009). This is because these different uses of dogs allow us to study the impact of lifestyle on their aging processes.

1.6 Thesis structure

In the following chapters we employed a multidisciplinary approach to investigate the dynamics of dog ageing. The research was developed in three main themes: first the methodological validation of the use of relative telomere lengths for dog welfare assessments and the examination of possible association between dogs' faecal glucocorticoids metabolites and dog's relative telomere length (Chapters 2 and 3); second, the investigation of dogs' relative telomere length and the dynamics of dog telomere length over a year (Chapters 4 and 5); and, lastly, the perception of a dog's apparent age from photographs evaluated by specialists and volunteers (Chapter 6).

The current research was approved by the University of Salford Science and Technology Ethics Panel (ethics application STR1617-22). The decision to sample using a non-invasive method led us to the validation of buccal swab use to collect DNA from buccal cells for telomere analysis, instead of the standard sampling method: blood sampling (Chapter 2). One of the most frequently used method for animal welfare assessment is the measurement of faecal glucocorticoids metabolites (Schwarzenberger, 2007). Therefore, we examined if there was any association between dogs' faecal glucocorticoids metabolites and dogs' relative telomere length to determine if relative telomere length could be used to give information regarding stress (Chapter 3). After these methodological validations we proceeded to sample dogs with buccal swabs to investigate the association of relative telomere length with dogs' age, sex, breed and background (Chapter 4). Then we selected laboratory dogs to be sampled twice a year apart to investigate how relative telomere length varies overtime in response to a social enrichment intervention (Chapter 5). In the search for new simple tools for non-invasive animal welfare evaluations we explored

the possibility of using apparent age to assess a dog's quality of life. Dog photographs were assessed by specialists and volunteers and their predictions associated significantly with relative telomere length (Chapter 6).

Within these topics we discuss the complexity of relative telomere length, its potential to be implemented in animal welfare assessment, the positive impact of animal enrichment on telomere length and the use of a novel approach to assessing animal welfare: apparent age evaluations. The aim of this research is to improve the welfare of dogs in shelters, police canine units, laboratories and pets by developing new tools and approaches to measure dog welfare.

Chapter 2 Validating the use of oral swabs for telomere length assessment in dogs

2.1 Introduction

Telomeres are defined as regions of DNA–protein complexes located at the ends of linear eukaryotic chromosomes whose function is to protect the DNA's integrity during cell division (Epel et al., 2004). In general, the telomere complex comprises double-stranded non-coding DNA sequences, usually consisting of tandem repeats of the nucleotides TTAGGG (Pat Monaghan & Haussmann, 2006). During cell division the telomere is not fully replicated, resulting in telomere shortening with every replication. Eventually it will reach a minimum length preventing any further cell division and therefore its protective function will trigger cell senescence (Nasir et al., 2001). Telomere length reduction is natural and a consequence of cellular division, aging, senescence and for this reason is used as a biological clock (Bateson, 2016). Studies have shown that fast telomere shortening (attrition), and short telomeres are related to cellular damage, making telomere lengths (TL) a valuable indicator of cellular aging and physiological stress (Aydinonat et al., 2014). Therefore, shorter telomere lengths are associated with chronic stress and diseases of old age, TL is of great interest in welfare assessment and healthy ageing studies (Woody, Hamilton, Livitz, Figueroa, & Zoccola, 2017).

TL may be assessed by a wide range of methods such as the Southern blot that measure Telomere Restriction Fragments (TRF), Flow-FISH cytometry and quantitative PCR (qPCR), which measures relative Telomere Length (rTL) (Cawthon, 2002). TRFs analysis by Southern blot is the gold standard method, and directly es-

timates the average telomere length in kilobases, whereas Flow-FISH method results are expressed as mean fluorescence intensity and the average telomere length are translated into kilobase estimates. (Gutierrez-Rodrigues, Santana-Lemos, Scheucher, Alves-Paiva, & Calado, 2014; Mckevitt, Nasir, Devlin, & Argyle, 2018). The measurement of telomere length using qPCR is determined by the average of telomere sequences content (nucleotides) in a sample using the ratio of telomere repeat copy number from a DNA sample to control gene (T/S ratio) (Cawthon, 2002). This technique has advantages over the others when it comes to cost, simplicity and amount of DNA needed for the analysis (Abechuco, Soto, Pardo, Haussman, & Diez, 2013).

Relative telomere length (rTL) can be measured in any cell population from proliferating tissues with high quality undamaged DNA; in humans, rTL is extensively measured in leukocytes from whole blood, but it can also be measured from buccal, liver, kidney, heart, spleen, brain, skin, triceps (muscle), tongue, intercostal skeletal muscle, subcutaneous fat, and abdominal fat cells (Dlouha, Maluskova, Lesna, Lanska, & Hubacek, 2014).

Recent research links environmental factors such as psychological and physiological stress, heart diseases, obesity and smoking to premature telomere shortening and accelerated human aging (Arsenis et al., 2015; Ash, Smith, Knight, & Buchanan-Smith, 2018; Hadchouel et al., 2015; O'Callaghan & Fenech, 2011; Zahran et al., 2015) . Recent studies show that poor animal welfare related to 'bad stress' (i.e. distress) is also associated with shorter telomeres in numerous non-human animal species (Ash et al., 2018; Aydinonat et al., 2014; Bateson, 2016).

Animal welfare is of global concern, not only from an ethical point of view but because it has significant negative financial impacts on the economies of animal industries (Mason & Latham, 2004). Animal welfare assessment usually considers general health and display of typical behaviours as welfare parameters, which can be measured through observations and physiological examinations, this can be time-consuming and expensive. (Broom, 2008). Traditionally, health checks were conducted using blood samples, however, capturing, containing and handling the animal during sample collection may stress the animal (Palme, 2012).

The length of telomere is traditionally assessed from blood samples, however, obtaining blood for research purposes would require a scientific licence, is costly, invasive to the animal, requires trained veterinarians, laboratory expertise and infrastructure for sample processing and storage (Thomson, Brown, & Clague, 1992). Studies have shown advantages of collecting saliva and buccal cells through swabs as they are more cost effective, stable at room temperature, collection does not require expertise and is less invasive, especially with trained animals (Feigelson et al., 2001). Despite the use of buccal swab cells and toe nails as a source of non-invasive DNA samples, there is no study describing the use buccal swabs for assessing rTL in any non-human species (Oberbauer et al., 2003).

Ensuring an animal's well-being in scientific studies involves choosing methodologies, which cause none or minimal distress to the subject. The development of more non-invasive methodologies is paramount for the animal welfare evaluation of pets, shelter, laboratory and captive animals. It is imperative that we have sampling methods that do not disturb the subject and at the same time allow for sample collec-

tion and a continuous monitoring of subjects' well-being. As the association of the relative telomere length from different body cells were already established we predict that the same association will occur with dog samples from different tissues. Thus, the present study addresses gaps in the scientific literature by measuring and comparing rTL from samples collected from two different tissues of healthy domestic dogs (*Canis familiaris*).

2.2 Objective

Investigate whether dogs' telomere lengths vary between samples collected from dried blood and from oral swab.

2.3 Justification

Presently, protocols for analysis of telomeres are typically performed with blood samples (Cai et al., 2015; Cawthon, 2002; Fick et al., 2012; Goyns, 2002). The intent of this research is to validate buccal cell swab sampling as a non-invasive and effective method for assessing telomere length in dogs. The initial intention was to only use oral swab sampling during data collection, but blood samples were necessary for validating the novel sampling approach, therefore a collaboration was established with a veterinary clinic for obtaining dogs' blood samples (Vetmaster, Veterinary Clinic, Belo Horizonte, MG, Brazil) (Stout et al., 2017).

The blood samples were not collected exclusively for our study, they were collected in routine veterinary exams requested by the owners, and all efforts were made to minimise suffering to the animals in these exams. All owners involved were

clarified about the project's objectives and received a project invitation letter, an information sheet and a consent form (Appendices 1, 2 and 3).

2.4 Methods

2.4.1 Ethical statement:

The data for this project were collected under ethical approval number: STR1617-22 conceded by The University of Salford Ethic Committee (Appendix 4). All biological material collected for this study was authorised under license number: ITIMP16.1096 (Appendix 5).

2.4.2 Samples

Samples were obtained from 25 dogs, 17 females and 8 males, ranging from 1 to 13 years old.

2.4.2.1 Dried whole blood spot collection

Sub-samples of venous blood that were collected during routine veterinary exams requested by the owners were donated for the research. Veterinarians also collected an oral swab from the same individual, at the same time, to allow a comparison between TL from blood samples and from oral swabs.

A drop of venous blood collected from each individual was fixed in an Indicating FTA™ micro card (GE Whatman, Maidstone, Kent, United Kingdom), air dried, stored in sealed plastic bags and kept at room temperature until further processing. (Proboste et al., 2015) (*Figure 5*).

Figure 5 Dried drop of whole blood fixed on an Indicating FTA™ micro card

2.4.2.2 Swab collection:

The swab was placed against the inside surface of the canid's cheek. Saliva and tissue were collected by rolling the Isohelix Buccal Swab (Cell Projects, Kent, UK) firmly against the cheek (Chang et al., 2007). A Dri-Capsule (Cell Projects, Kent, UK) was added to each swab tube to allow the sample to be stored at room temperature without DNA degeneration (Küffer et al., 2016) (*Figure 6*).

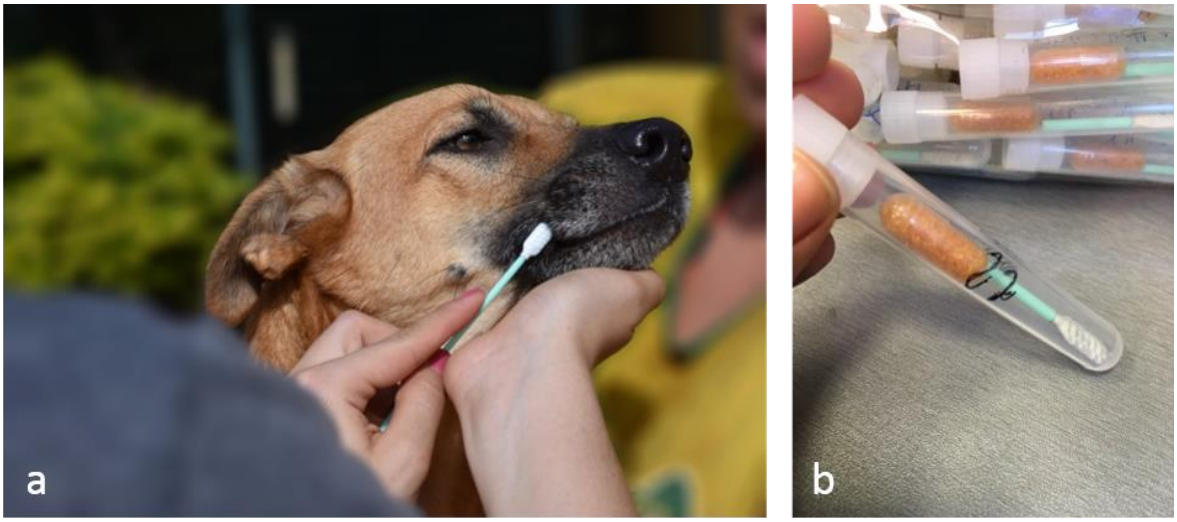

Figure 6 a) Swab sampling dog for DNA in a shelter in Brazil b) Swab stored after dogs' buccal cell collection kept under room temperature.

2.4.3 DNA extraction

The DNA extraction from the blood and swab samples was performed using DNeasy® Blood & Tissue Kit (Qiagen) following the manufacturers' protocol with a few adjustments (Appendix 6).

2.4.4 DNA quantification

Concentration of DNA extracted from dried whole blood spot and swab samples was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Then all samples were diluted to a working stock concentration of 50 ng/µL in PCR-grade water and single-use aliquots were kept frozen at –30 ºC.

2.4.5 Primer validation

For the relative telomere length measurement, we tested primers described in literature (*Table 1*).

Table 1 Primers described in literature for relative telomere length estimation tested in qPCR assay

Primers trials		
Primers	Target gene	Reference
Tel1, Tel2	Telomere	Cawthon, 2002
Telb1, Telb2	Telomere	Giradeaudeu, 2016
Telc, Telg	Telomere	Cawthon, 2009
36B4F, 36B4R	36B4	Cawthon, 2002
36B4mF, 36B4mR	36B4	Buddhachat, 2017
Albu, Albd	Albumin	Cawthon,2009
18SF, 18SR	18S	Giradeaudeu, 2016

We took into consideration Richard Cawthon's (personal communication, 21 of February 2018) advice to work with albumin as the gene reference for the relative telomere measurement because albumin have been used previously for measuring telomere from human samples (Cawthon, 2009). Since there were few mismatches from the human primer compared to dog, three primers were design specifically for canine albumin. Primers had similar thermodynamic properties, so they would be able to be used in the same PCR cycling conditions (*Table 2*).

Table 2 Albumin Primers Designed for qPCR trial with domestic dogs

Albumin Primers Design		
Primers	Sequence	Size
Alb1_F	AGACATCCGTACTTCTACGCCC	22
Alb1_R	GCAGCACTCCGCAAAGACTC	20
Alb2_F	TGAGCCAGCGATTTCCCAAAG	21
Alb2_R	TGGCAGCATTCCTTGTGGAC	20
AlbcClamp F	CGGCGGCGGGCGGCGCGGGCTGGGCGGagacatccgtacttctacgccc	49
AlbcClamp R	GCCCGGCCCGCCGCGCCCGTCCCGCCGgagtctttgcggagtgctg	47

After testing more than seven primer sequences for telomere and reference gene, the ones that showed the best performance for dog swab samples for telomere gene were Telg and Telc from Cawthon (2009) and for the reference gene 18S were 18S-F and 18S-R from Giraudeau and colaborators (2016). As multicopy genes were already used in literature for measuring relative telomere length and showed the same consistence as a single copy gene, the 18S was used the reference gene and primers were used at the same concentration (800nm) (*Table 3*) (Wang et al., 2013). The performance of each primer on each reaction was calculated by the Rotor-Gene Q Series v.2.3.1 software and were between 98% and 100% efficient.

Table 3 Primers used for measuring domestic dogs' relative Telomere Length in qPCR assay

Primer	Primer sequence	Target gene	Reference
telg	5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'	Telomere	(Cawthon, 2009)
telc	5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'	Telomere	(Cawthon, 2009)
18S F	5'-GAGGTGAAATTCTTGGACCGG-3'	18S	(Plot et al., 2012)
18S R	5'-CGAACCTCCGACTTTCGTTCT-3'	18S	(Plot et al., 2012)

2.4.6 qPCR assay

A pool from the 25 swab samples (conc. 107.2 ng/µl) and one for the dried whole blood spot (conc. 190.9 ng/µl) was produced. Each qPCR batch (i.e. run) consisted of two components: i) a tenfold point serial dilution prepared from pool of DNA from the swab pool or from the dried whole blood spot, which was used to construct the standard curve and formed the basis of each primer optimization and quality control; ii) no template controls (NTC), which allowed for detection of potential contami-

nation and/or primer dimer formation. This was done for the telomere gene and the reference copy gene. Each step of the dilution series and the NTC were run in triplicate in each batch (Olsen, Bérubé, Robbins, & Palsbøll, 2012).

Primers were designed according to literature, telomere primers telg, (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), telc, (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3') from (Cawthon, (2009); and 18S primers, 18S sense (5'-GAGGTGAAATTCTTGGACCGG-3'), 18S antisense (5'-CGAACCTCCGACTTTCGTTCT-3') for the reference gene from Plot, Criscuolo, Zahn, & Georges (2012). Quantitative PCR reactions were performed using monochrome multiplex assay adapted from Olsen, Bérubé, Robbins, & Palsbøll, (2012). Master mix reactions were prepared following O'Callaghan & Fenech (2011) master mix protocol *(Table 4).* Amplifications were performed in a Rotor - Gene ® Q cycler (QIAGEN) with the Rotor-Gene Q Software ver. 2.3.1 using the manufacturer's 0.1 ml strip tubes and caps. Conditions were 95°C for 15 min, 2 cycles of 94°C for 15 s and 49°C for 15 s, 40 cycles of 94°C for 15 s, 62°C for 10 s, 74°C for 15 s with signal acquisition, 84°C for 10 s, 88°C for 15 s with signal acquisition, and concluded with a melting curve ramping from 72°C to 95°C, rising by 0.5°C in steps of 30 s (Olsen et al., 2012). Each run had a standard curve calculated by the Rotor-Gene Q Series v.2.3.1 software and the dilution factors of the standards corresponding to the telomere and reference gene amounts and each sample.

Table 4 Representation of master mix preparation for the qPCR reaction (O'Callaghan & Fenech, 2011)

Master mix preparation for relative telomere length assay		
Reagents	Volumes for one sample (µl)	Final concentration
Power SYBR Green master mix (2x)	10	1x
Primer (telomere-fwd (2 µM))	1	0.1 µM
Primer telomere-rev (2 µM)	1	0.1 µM
H_20	4	-
DNA (5 ng/µl DNA)	4	20 ng total

2.4.7 Measuring relative telomere length (RTL)

The measurement of the relative telomere length was calculated by using an adaptation of the qPCR method described by Cawthon (2002), using a multi-copy gene instead of a single copy gene as the reference gene as validated previously by Wang et al. (2013). The calculation consisted in the ratio of telomere repeat copy number (T) to reference gene copy number (S). As each experimental sample was assayed in duplicate, two T/S results were obtained for each sample, if the duplicates values differed from each other we did a second assay for the sample; the final reported result for each sample in a given run was the average from each duplicate (Cawthon, 2002).

2.4.8 Statistical Analysis

We tested the normality of T/S ratios from each sample using Kolmogorov-Smirnov tests, differences between the T/S ratio means from blood and swab samples with paired t-test and conducted Pearson correlations to assess the concordance of T/S ratios across samples from dried whole blood spot and saliva. All statistical analyses were performed using Minitab® 18.1.

2.5 Results

2.5.1 DNA quantity and quality

Results are summarized in Table 5. Dried Blood Spot DNA yield had a mean DNA concentration of 196.82 ng/µl [range: 23.1 - 397.3 ng/µl] and was greater than swab DNA yield with 115.37 ng/µl mean DNA concentration [range: 18.40 – 240 ng/µl]. All samples were successful in providing adequate DNA quantities, despite this Dried Blood Spot had two samples with low concentration quantified (*Figure 7*).

Table 5 Mean DNA yield and percent Standard Error (SE) for each dog sample collected with swab and dried blood spot

Sample type	Mean DNA yield (ng/µl)	Range (ng/µl)	Mean SE (%)	N
Swab	115.37	18.4 – 240.0	10.92	25
Dried blood spot	196.82	23.1 - 397.3	7.36	25

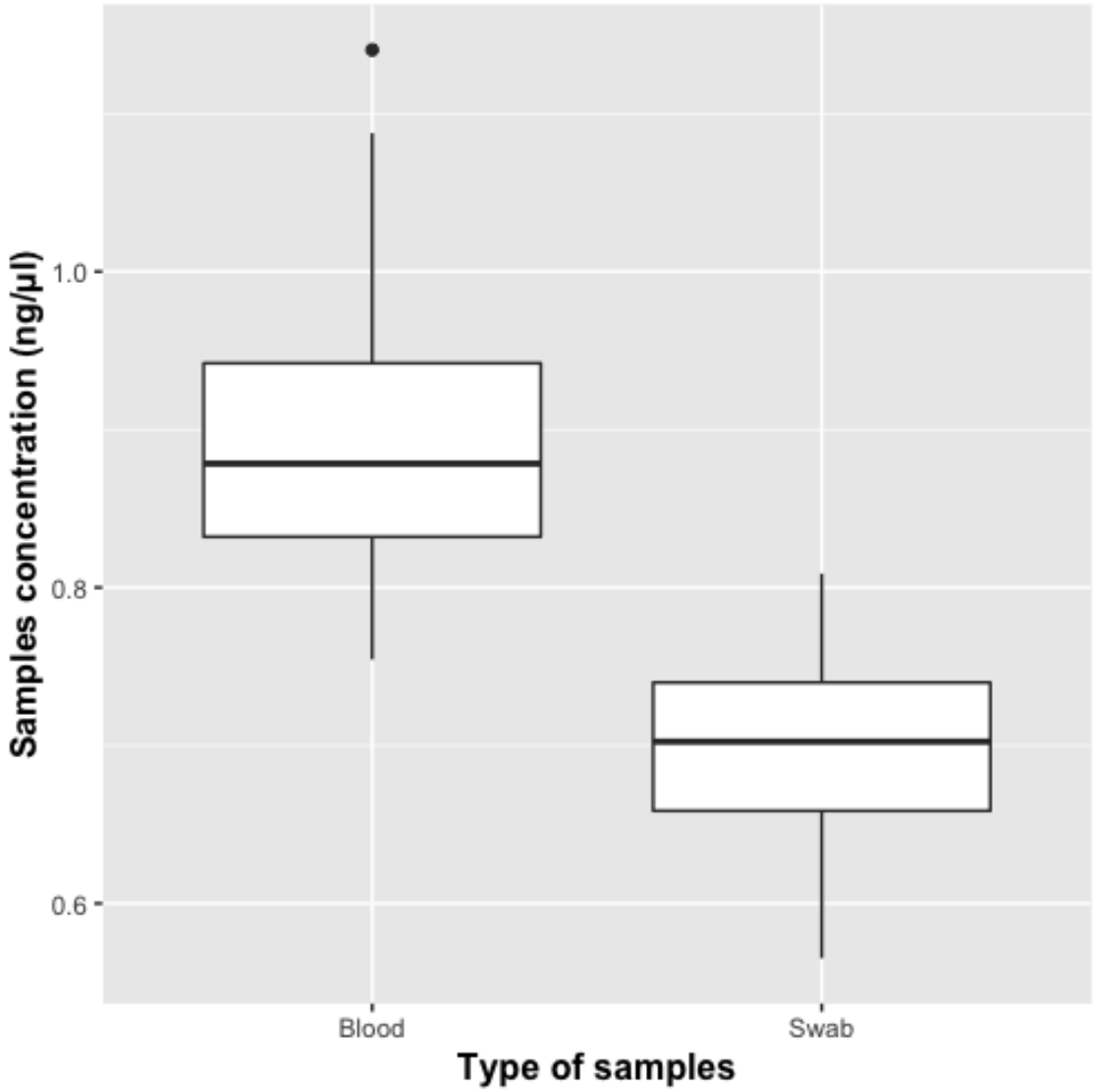

Figure 7 Median DNA concentration (ng/µl) for each collection method. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers;

2.5.2 Swab and Dried Blood Spot rTL were significantly correlated

Relative telomere length data were normally distributed (assessed using Kolmogorov-Smirnov tests). The associations between in rTL dried whole blood spot and saliva can be seen in Figure 8. Swab T/S and dried whole blood T/S were significant and positively associated (Pearson Correlation $r = 0.42$, $p < 0.05$).

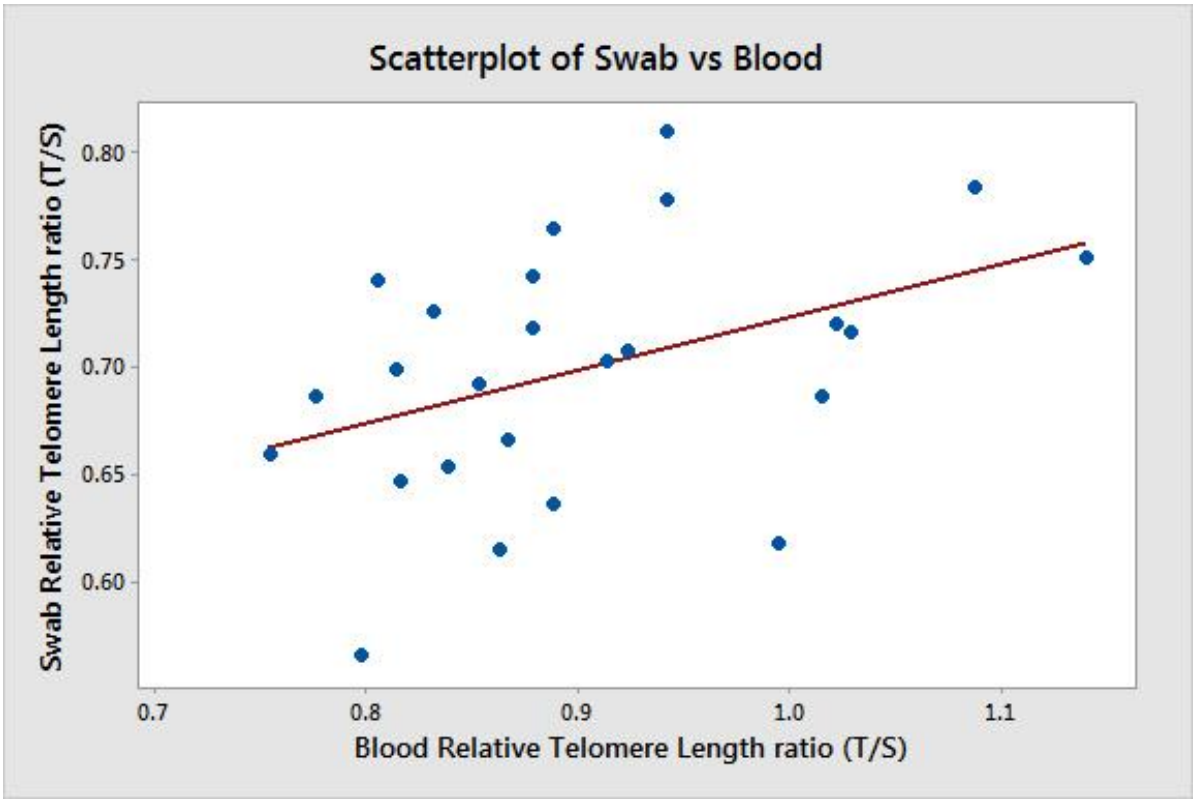

Figure 8. Telomere length (TL) was significantly associated across sample types. T/S in dry spot blood and dried blood spots (DBS) had a medium strength positive correlation ($r = 0.42$, $p < 0.05$).

2.6 Discussion

The current study indicates that oral swab samples can be effectively used to measure TL, and that the method is a viable alternative to invasive venous blood sampling. Obtaining swab samples from the buccal cavity of dogs is a promising sampling method, as it is non-invasive and quicker to perform than standard blood sampling.

Few studies investigate the use of non-invasive sample collection for obtaining relative telomere length in humans (Goldman et al., 2018; Stout et al., 2017). Differently from Goldman et al. (2018) that obtained greater DNA concentration from

oral swabs our dried blood spots yielded a greater DNA concentration when compared to the swab samples, but consistent with our prediction and as in the mentioned study a positive medium strength significant correlation between swab and blood samples was found.

When choosing a sampling method for assessing animal welfare the ease and the cost effectiveness must be considered not only to avoid stressing the individuals' during collection but to increase the number of samples collected at an affordable cost and effort. The present study provided evidence demonstrating the concordance of relative telomere length across dried blood spot and buccal swab from domestic dogs. Therefore, swab sampling can be used as a sample collection method not only for pet dogs, which the owners can collect their dogs samples, but for animals in shelters and laboratory facilities (Laule, Bloomsmith, & Schapiro, 2003; Oberbauer et al., 2003). Positive reinforcement training can be used to collect swab samples with as little stress for the animals as possible to produce most reliable research results and to protect and enhance the well-being of the animals (Laule et al., 2003).

Thus, this chapter has validated buccal cell samples from dogs for relative telomere length assessments and as such this approach was used in the rest of my PhD projects.

Chapter 3 Validating telomere length as a marker for animal welfare in domestic dogs

3.1 Introduction

Time as a parameter is a very important aspect to consider when evaluating animal welfare because it will determine one's hypothesis, methods and sampling regime. If you are interested in an animal's immediate response to a stimulus researchers need to evaluate changes in behaviour or measure specific hormones in blood or saliva (Afonso et al., 2012; De Palma et al., 2005; Hennessy, Williams, Miller, Douglas, & Voith, 1998). To evaluate a chronic effect from a stimuli studies can use changes in behavioural states, appearance of abnormal behaviour or long term hormone secretion measured in faecal samples, effects on immunity or reproductive success (Mason & Latham, 2004; Owen et al., 2004; Vasconcellos et al., 2011). The concept of quality of life was developed to understand how an animal's experiences impact their lives; it refers to an impact on an individual over a time-scale longer than right now or a few days (Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Phillips, et al., 2007).

Another concept of animal welfare measurement considering time scale, which has not been used until recently, is lifetime cumulative experience (i.e., the sum of an animal's positive and negative welfare experiences over its life to the time of sampling; (Bateson, 2016)).

Animal Welfare is the science that studies animals' quality of life and works mainly with two conceptual frameworks: individuals' biological functioning or physiology, and behaviour (Hosey, Melfi, & Pankhurst, 2009). Behaviour can be assessed through, enclosure use, activity budget, preference tests or presence of abnormal behaviour whilst animals' physiology can be evaluated by body condition score, life expectancy, immune-system functioning, reproductive success, stress hormones secretion, heart-rate variability, etc (Rees, 2015).

The way that animals cope with challenge and environmental change is highly related to their stress response and measuring hypothalamic–pituitary–adrenocortical (HPA) axis activity is the standard approach to the study of stress and welfare in farm animals (Mormède et al., 2007; Sheriff et al., 2011). Through measures of glucocorticoid metabolite (GCM) levels (a stress hormone metabolite), it is not only possible to measure an animal's welfare but also to define animal management or husbandry strategies (Teixeira, Azevedo, Mendl, Cipreste, & Young, 2007). The reference technique to measure glucocorticoid hormones is the use of blood plasma samples (Hennessy et al., 1998). Several alternative methods such as the measurement of corticosteroids in saliva, urine or faeces have been developed to overcome the stress induced by blood sampling procedures (Mormède et al., 2007). Generally, stress hormones circulating in plasma are metabolized by the liver and then excreted in urine, from the kidneys or as bile into the gut (Touma & Palme, 2005). The major advantage of faecal sampling , from an animal welfare perspective, is that hormone metabolites can be collected without capturing and disturbing the animal (i.e., the sampling does not affect what you are trying to measure; Hulsman et al., 2011).

To measure faecal glucocorticoid metabolites (FCM) there is a time interval between stress events and the appearance of the respective signal in the faeces. This interval is in general species-specific because it depends on the intestinal transit time from the duodenum to the rectum, and must be considered when studying endocrine patterns (Touma & Palme, 2005). Studies conducted with dogs showed that this lag time is between 20-24 hr for peak glucocorticoid concentrations in faecal material (Schatz & Palme, 2001). Currently, this method is being used to investigate questions about hormone–behaviour interactions, reproduction, animal welfare, and conservation (Möstl, 2014; Teixeira et al., 2007; Vasconcellos et al., 2011). It is worth pointing out that this method provides a mean stress level (and not an instantaneous one) over a period of several hours (Möstl & Palme, 2002b). Thus, this method would be useful to determine the impact of a stressor, which impacts an animal over a timeframe of several hours (e.g. sound pollution). Whereas, it would not detect a short-term stressor such as an on-off sudden noise; this could be detected by blood or saliva measurements of cortisol, heart-rate variability or behavioural responses (e.g. fear response).

Stress and the hormones secreted during stressful situations are relevant to many aspects of health. Evidence suggest that stress and glucorticoids have a significant impact on the nervous system's health and can increase mortality due to immune disorders, such as autoimmune diseases, cardiac diseases, cancer and infections (Sapolsky, 1999). Furthermore, a lifetime of elevated stress will also impact the body at a molecular level, causing accelerated ageing in cells (Dreschel, 2010). Whereas, low levels of cortisol might be more indicative of healthy ageing (Noordam et al., 2012).

Domestic dogs, just like humans, also suffer from stress when experiencing poor welfare (Beerda, Schilder, Van Hooff, De Vries, & Mol, 2000; Cai et al., 2015). Poor housing conditions, boredom, loneliness, aversive training techniques, the addition or loss of a family member or pet and an uncontrollable or unpredictable social environment are examples of situations that may act as stressors for pet dogs (Beerda et al., 1997).

For shelter dogs confinement (i.e. limited control over their ability to explore their environment) is believed to be the main source of stress, however, there are many other potential stressors such as unpredictable noise, change in exercise routines, separation from their previous owner that will also provoke chronic HPA activation (Hennessy, Williams, Miller, Douglas, & Voith, 1998).

Aside from being companion animals, dogs are used as well in working environments in military tasks, human assistance and other functions that can expose them to various stressful situations. Military dogs, for example, are trained to act in chaotic environments with loud noises such as explosions and to deal with human aggressors (Horváth et al., 2007; Whitmarsh, 2005).

Telomeres are terminal DNA–protein complexes at the chromosome ends that act as ‘protective caps’ (Sanders & Newman, 2013). Studies show that telomeres shorten with age, contributing to organismal aging and for this reason telomere length has been found to predict lifespan (Küffer et al., 2016). The reasons for telomere shortening are still not fully comprehended, however, many studies propose that this mechanism is influenced by exposure to chronic stress and lifestyle.

Chronic stress increases oxidative stress damage to neurons, decreases antioxidant enzymes and causes DNA damage (Choi et al., 2008). For example, a study showed that shortened telomere length (TL) is a significant marker for major depression and presented an association between TL and cortisol reactivity to stress in girls (Gotlib et al., 2015). Other factors – including financial problems, obesity and smoking are associated to reduced telomere length in humans (Agrigoroaei et al., 2017; Valdes et al., 2005). In nonhuman animals we have studies associating TL shortening to stressful situations such as exposure to infectious disease or social isolation, but none of them simultaneously investigated the effect of stress hormones in their studies, the advantage of using both methodologies is that the findings can help identify the potential mechanism for stress-associated telomere length attrition (Aydinonat et al., 2014; Ilmonen, Kotrschal, & Penn, 2008).

Animal welfare science has developed into a robust scientific field due to the use of various indicators from different scientific disciplines (Fraser et al., 1997). By using a mixture of physiological and behaviour parameters studies have provided a better understanding of the causes of poor wellbeing through the use of cross validation studies (Hewison, Wright, Zulch, & Ellis, 2014; Part et al., 2014; Salas et al., 2016). The present study addresses gaps in the scientific literature by measuring how relative telomere length (rTL) and cortisol levels of domestic dogs (*Canis familiaris*) covary to validate the use of rTL as a non-invasive technique for assessing animal welfare but, importantly, considering cumulative life-time experiences.

3.2 Objectives

Investigate whether dogs' telomere length present in swab cells co-varies with glucocorticoid levels (i.e. short-term stress levels)

3.3 Justification

Currently when animal welfare is assessed by physiological parameters, typically it is through measuring cortisol levels (Glenk et al., 2013; Hennessy, 2013; Möstl, 2014; Sapolsky, 1999). The intent of this research is to validate another non-invasive method for welfare assessment in domestic dogs: the relative telomere length analysis. The association of rTL and cortisol levels is already established for humans; however, animal welfare studies using rTL has not investigated this association yet (Bateson, 2016; Gotlib et al., 2015).

3.4 Methods

3.4.1 Subjects

We collected samples from two different groups of domestic dogs in Brazil: 13 German Shepherd dogs from a commercial kennel (5 males and 8 females); and 13 mixed breed dogs (7 males and 6 females) laboratory housed during seven consecutive days. We chose to work with these two dogs' groups for several reasons: all dogs within the groups are kept in individual kennels, which facilitates faeces collection; and, therefore, they are kept under the same treatment, diet and exercise routine. As all dogs within the groups have the same background and husbandry, the cortisol variations are mainly related to individual differences.

3.4.2 Dog housing and husbandry

Dogs from laboratory had their kennel cleaned twice a day and were fed after every cleaning. They did not routinely have exercise or social activities (*Figure 9*).

Dogs from the commercial kennel had their facilities cleaned twice a day and were also fed twice a day. They were walked every day and had training activities at least three times a week (*Figure 10*).

Figure 9 Facilities of laboratory housed domestic dogs (Canis familiaris)

Figure 10: Facilities of the commercial kennel domestic dogs in Brazil (Canis familiaris)

3.4.3 Collection of faeces:

Seven fresh faecal samples were collected for each dog. Dogs defecate once or twice a day; the samples were collected in the morning and this reflects adreno-cortical activity in the previous day (De Palma et al., 2005; Schatz & Palme, 2001).

Every faecal sample was mixed immediately after collection to ensure sample homogeneity, stored in 5-mL screw cap tubes marked with date and frozen (-20° C) until extraction at Pontifical Catholic University of Minas Gerais, Brazil (Palme & Möstl, 1997; Palme, 2005; Vasconcellos et al., 2011).

3.4.4 Hormone Extraction

Prior to analysis, steroid metabolites were extracted from the faeces (FCM) using methods from protocols developed by Möstl (2009), and Schatz and Palme (2001). Following the protocol developed by Möstl (2009) 0.5 grams of wet faecal samples was added to 5mL 80% methanol (premixed; or 4 ml 100% methanol and 1

ml water). Then samples were mixed in a multi vortex for 30 min, centrifuged for 15 min (2500 g), and lyophilized before being sent for enzyme immunoassay.

3.4.5 Enzyme Immunoassay

The amounts of FCM were determined using a cortisol enzyme immunoassay as described by Palme and Möstl (1997). Schatz and Palme (2001) successfully validated this assay with dogs' faeces to monitor changes in adrenocortical activity. Concentrations of faecal metabolites of glucocorticoids measured by enzyme immunoassay were expressed as nanograms per gram of wet faeces (Vasconcellos et al. 2011). The enzyme immunoassay was performed in collaboration with Prof Rupert Palme's laboratory at Department of Biomedical Sciences, University of Veterinary Medicine, Vienna, Austria.

3.4.6 Swab collection

To associate cortisol levels and telomere length the dogs were orally swab sampled to collect buccal cells (i.e. DNA) following the sampling protocol as described previously in Chapter 2.

3.4.7 DNA extraction and quantification

The DNA extraction from the swab samples was performed using Buccalyse DNA Release Kit DNA following the manufacturer's protocol. Concentration of DNA extracted from swab samples was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

3.4.8 qPCR assay

A pool from the laboratory dogs' samples (conc. 94.2 ng/µl) and one for the kennel dogs' samples (conc. 139.6 ng/µl) was produced. Each qPCR batch (i.e. run) consisted of two components: i) a tenfold point serial dilution prepared from each pool of DNA, which was used to construct the standard curve and formed the basis of each primer optimization and quality control; ii) no template controls (NTC), which allowed for detection of potential contamination and/or primer dimer formation. This was done for the telomere gene and the reference copy gene. Each step of the dilution series and the NTC were run in triplicate in each batch (Olsen, Bérubé, Robbins, & Palsbøll, 2012).

The primers, qPCR reaction mix and conditions were performed as described previously in Chapter 2.

3.4.9 Measuring relative telomere length (RTL)

The measurement of the relative telomere length was calculated by using an adaptation of the qPCR method described by Cawthon (2002), as described previously in Chapter 2.

3.4.10 Statistical analysis

In addition to the swab and faecal samples information regarding the dogs' sex, age and health status were also collected.

All data were tested for normality (Kolmogorov–Smirnov Test) and the results of all statistical tests were considered significant at $p < 0.05$. Generalised linear models (GLM) with Gaussian distribution (Fields, Miles, & Field, 2012) were used to

identify which of the aforementioned factors had impact on a dog's relative Telomere Length (rTL). To define the best model, we ran GLM considering the interaction of response factors and explanatory variable. All GLM models started with all variables included and then the non-significant variables were removed one by one using the drop1 function. Then each model was compared using the Akaike's information criterion (AICc), where the best fit model has the lowest AICc value, and then the most explanatory model was chosen.

In the first model we investigated if a dog's sex, age, group (from the laboratory or from the commercial kennel) and mean cortisol level (response variables) had any influence on a dog's relative Telomere Length (explanatory variable). The second and third models considered the dogs groups separately when investigating if sex, age and mean cortisol concentration (response variables) had any influence on the relative Telomere Length (explanatory variable). All statistical analysis were run under the platform Studio R (RStudio Team, 2016).

3.5 Results

3.5.1 Model 1: Factors that impact on a dog's relative Telomere Length

The residuals of relative Telomere Length were normally distributed, so the first GLM model was performed with Gaussian distribution including all background factors and using the function drop1 was used to remove all non-significant variables; this achieved the optimal model were only the explanatory factors sex, age group were left. The factor Cortisol metabolites was excluded from the ideal model. The chosen model showed that neither sex ($t = 0.283$, $df = 25$, $p > 0.05$), or age ($t =$

1.128, df = 25, p > 0.05), had any effects on a dog's relative Telomere Length. However, the group factor had a significant effect on relative Telomere Length (from the laboratory or from the kennel). GLM results for model 1 are summarized in Table 6.

Table 6 Generalised linear model result for the effects of sex, age, group and cortisol levels on relative telomere length of domestic dogs from two different backgrounds: a kennel and a laboratory [1]

Parameters	Estimate±SD	t value	P
Intercept	0.7797 ±0.0336	23.178	<0.0001**
Sex[ab]	0.0090±0.0320	0.283	0.7795
Age	0.0062±0.0055	1.128	0.2712
Group[c]	-0.1213±0.0318	-3.817	<0.0001**

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -48.83

[a]Explanatory variables included in the full model: Sex, Age, Group and Cortisol metabolites

[b] Male or Female: Male is the reference group

[c] Kennel or Laboratory: Laboratory is the reference group

** P ≤ 0.001

3.5.2 Model 2: Factors that impact on a kennel dog's relative Telomere Length

Since model 1, included dogs from two different groups, which indicated an effect on dogs' relative telomere length we proceeded to investigate this effect considering dogs from the different groups separately. Numerical data were normally distributed, the second GLM model was performed with Gaussian distribution including all background factors and using the function drop1 was used to remove all non-significant variables, in the achieved optimal model the only explanatory factor left was age. Factors Sex and Cortisol metabolites were excluded from the best fit model, showing that neither sex (t = 0.033, df = 12, p > 0.05), age (t = 1.161, df = 12, p >

0.05) nor basal cortisol (t = - 0.064, df = 12, p > 0.05), had any effects on a kennel dog's relative Telomere Length. GLM results for model 2 are summarized in Table 7.

Table 7 Generalised linear model result for the effect of sex, age and basal cortisol levels on relative telomere length of dogs from a kennel [1]

Parameters	Estimate±SD	t value	P
Intercept	0.7678 ±0.0414	18.519	<0.0001**
Age [a]	0.0103±0.0080	1.284	0.2260

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= - -18.743

[a] Explanatory variables included in the full model: Sex, Age and Cortisol metabolites

** P ≤ 0.001

3.5.3 Model 3: Factors that impact on a laboratory dog's relative Telomere Length

Continuing the analysis, in the next model we considered only the results from the laboratory dogs. The third GLM model was performed with Gaussian distribution including sex, age and cortisol metabolites as explanatory factors, after using the function drop1 only the explanatory factors age and cortisol metabolites were left. The factor sex was excluded from the ideal model. Age had no significant effects on laboratory dog's relative Telomere Length (t = -0.332, df = 12, p > 0.05). Cortisol metabolites showed significant association with the dogs' relative Telomere Length (t = -2.733, df = 12, p < 0.05). GLM results for model 3 are summarized in Table 8 (*Figure 11*). This association explains 50.3% of the variation in relative Telomere Length indicating that it is a biologically important factor.

Table 8 Generalised linear model result for the effect of sex, age and basal cortisol levels on relative telomere length of dogs from a laboratory [1]

Parameters	Estimate±SD	t value	P
Intercept	0.7741 ±0.2578	29.63	<0.0001**
Age [a]	-0.0015±0.0047	-0.332	0.7470
Cortisol metabolites	-0.0108±0.0039	-2.773	0.0190*

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -48.83

[a]Explanatory variables included in the full model: Sex, Age and Cortisol metabolites

*P ≤ 0.05

** P ≤ 0.001

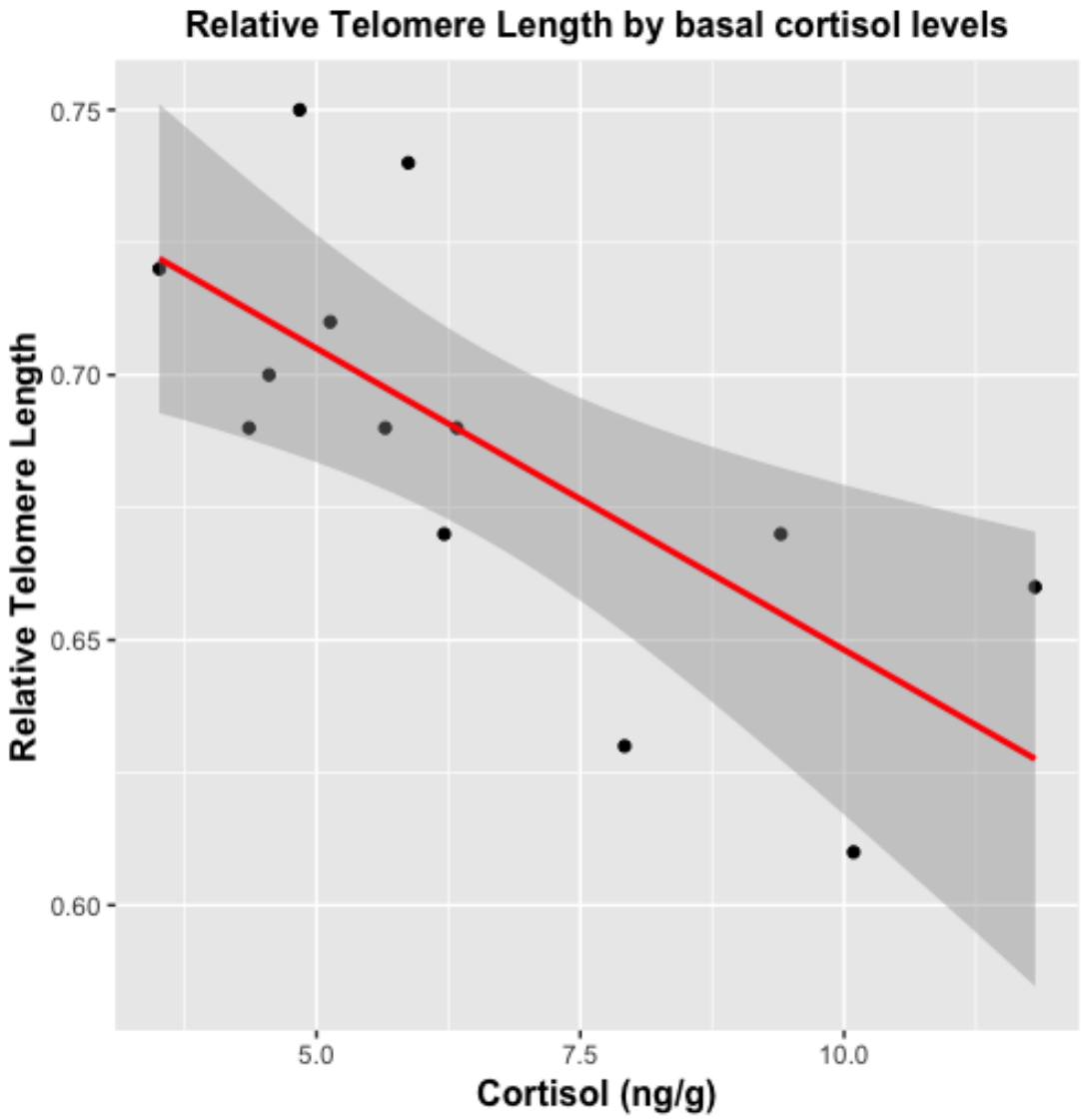

Figure 11 Correlation of basal cortisol levels and relative telomere length of laboratory dogs.

3.6 Discussion

This study showed a significant concordance of relative telomere length and basal cortisol levels from domestic dogs in a laboratory under barren housing conditions. Thus, cortisol data validated the relative Telomere Length data, but only when housing conditions are expected to have strong negative impacts on animal welfare.

When considering physiology to evaluate animal welfare faecal glucocorticoids metabolites (FGM) are one of the most used non-invasive methodologies, due to their simple collection, sensitive and precise results (Palme & Möstl, 1997). Although this technique is validated for various species many others, especially wild species, still need technique validation (Möstl, 2014). This validation is done through an ACTH challenge, which consists of injecting small doses of synthetic adrenocorticotropic hormone (ACTH) in to the subject, a hormone that stimulates the adrenal glands to release cortisol, and then measure the amount of cortisol produced in response (Holsboer, Gerken, Stalla, & Müller, 1985). This technique validation involves capturing and handling the animal, which can be a stressful event for the animal and is not always possible. It is important to emphasise that stress is only one of the many parameters that should be used to assess animal welfare, others such as life expectancy or cumulative life experiences are also important (Bateson, 2016). The other disadvantage of the FCM analysis is that to have a reliable result it is necessary to collect faecal samples prior, during and after a stressful stimuli or for at least seven consecutive days to obtain a basal cortisol level (Briefer Freymond et al., 2015).

Using telomere length from swabs to evaluate welfare circumvents the major drawbacks from FCM method because it can be collected a single time using and through positive reinforcement training (Ash et al., 2018; Westlund, 2014).

Our first statistical model only found an effect of the group factor on relative telomere length, cortisol had no significant impact. Until the present moment all studies regarding these associations were carried only with human subjects, where the

sample sizes ranged from 77 to 380 individuals (Choi et al., 2008; Gotlib et al., 2015; Noordam et al., 2012; Woody et al., 2017). Despite having small a sample size, this study is valuable for helping understand a telomere's length and cortisol associations in a non-human species.

When taking in consideration the dogs' group and analysing these separately, in the second model, the kennel dogs did not show that their telomere lengths were associated with cortisol levels. The kennel dogs have arguably a good quality of life, with exercise routine, since only a small number of dogs were sampled the effect of cortisol could have been masked. Studies human subjects that showed association between cortisol and telomere length had greater sample number, which could facilitate identification of this association (Choi et al., 2008). Thus, results in relation to kennel dogs may be a type 2 statistical error: a false negative (Fields et al., 2012).

The third statistical model used the samples of the dogs from a laboratory and in this group basal cortisol level showed a medium strength significant and negative correlation with relative telomere length. A possible explanation for this is that the laboratory environment was much poorer for animal welfare in comparison with the kennel environment. Studies have showed that poor housing conditions associated with laboratory housing can be a source of stress, due to sound pollution, spatial restriction, lack of stimulation, which provoke increasing levels of abnormal behaviour (Bonne Beerda et al., 1999; Clark, Rager, Crowell-Davis, & Evans, 1997; Sales, Hubrecht, Peyvandi, Milligan, & Shield, 1997).

In conclusion, the agreement between cortisol levels and relative Telomere Length for laboratory dogs increases scientific confidence in the use of the latter as a

measure of animal welfare. Furthermore, the present results may help elucidate the complex interaction between environment and endocrine system, which can both lead to precocial ageing due to telomere shortening. We should note the limitations of the current study, which is its relatively small sample size that can mask any effect of cortisol levels in individuals with fairly good welfare. Therefore, it is important in future research to investigate telomere length and cortisol associations considering sample size and aiming to obtain the same number of individuals from both sex, different ages and breeds.

Chapter 4 Evaluating dogs' lives through relative Telomere Length (rTL)

4.1 Introduction

Humans and dogs share an exceptional social relationship and the explanation for this is found in the dogs' domestication process, where the human–animal interaction benefited dogs as well as people (Marinelli et al., 2007). One result of this domestication is that numerous studies have shown how dogs are able to communicate with humans using diverse strategies that other non-human species cannot (Kaminski & Nitzschner, 2013). Dogs can read human emotion, gaze, movement, gesture and speech skills which certainly contribute to owners developing a strong bond with their dogs, and possibly for this reason they classify their dogs as companion animals (Albuquerque et al., 2016; Part et al., 2014; Téglás, Gergely, Kupán, Miklósi, & Topál, 2012; Virányi, Topál, Gácsi, Miklósi, & Csányi, 2004; Worsley & O'Hara, 2018). Dog ownership is associated with improvements in general wellbeing, increase in exercise levels, improved physical health, a confident psychological state and a decrease in stress levels (Headey, Na, & Zheng, 2008; Hoffman, Stutz, & Vasilopoulos, 2018; Thorpe et al., 2006). Similarly research has shown that dogs also benefit from this relationship, with more attached owners being able to predict and monitor better their companion's health and affective states (Schneider et al., 2010).

Although most studies have focussed on the benefits to humans in the human-dog relationship there is evidence of positive effects for dogs in this dyadic association, positive human interactions reduce the stress levels of dogs from animal shelters (Hennessy, Williams, Miller, Douglas, & Voith, 1998). Dogs show less signs

of stress when in human presence when in novel situations highlighting the attachment bond between dogs and humans (Tuber, Hennessy, Sanders, & Miller, 1996).

Studies have also been investigating the emotional attachment from dog's owner towards theirs dogs showing that dogs are often described as friends, companions or children, this close relationship can enable owners to consider more carefully the quality of life of their dogs, which was typically assessed only by veterinary surgeons (Marinelli et al., 2007; Mariti et al., 2012; Martens, Enders-Slegers, & Walker, 2016; Wojciechowska & Hewson, 2005).

Besides being a companion, dogs can also be working companion, mostly because of their sociability and trainability, they are able to play important roles on farms, in hunting, guarding, for military, searching, rescuing activities and also as assistance therapy dogs (Bryant, Dunham, & Overall, 2018; Kim, Oh, Hwang, Hur, & Lee, 2018; Lefebvre, Giffroy, & Diederich, 2009; Svartberg, 2006).

When conducting research, experiments conditions need to be extremely controlled for this reason laboratory dogs tend to live in a very stable environment regarding diet, housing, social interaction and husbandry (Hubrecht, 1993). Housing is one of the most critical features for laboratory dogs' welfare since enriched environments could affect the animal model influencing the research results in a uncontrollable way hence laboratory dogs are housed individually and usually have barren small housing (Spangenberg, Björklund, & Dahlborn, 2006). Nevertheless, contrary to common sense, a study shown that there was no evidence that enrichment increased variation in the data of animal experiments; actually enriched housing increased mice welfare without disrupting experiments (Würbel & Garner, 2007). Alt-

hough studies indicate that dogs find comfort in predictable environments and situations it is still debatable if the predictability of laboratory routines and practices are enough to counterbalance social isolation and confinement, which are factors that are related to poor welfare conditions (Amat, Camps, Le Brech, & Manteca, 2014; Glenk et al., 2016; Hubrecht, 1993; Spangenberg et al., 2006; Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Phillips, et al., 2007; Tuber et al., 1996).

Military dogs are intensively trained to work in loud environments, with various sounds, scents and different people, although trained to cope with this factors, all these stimuli play a negative role (Lefebvre et al., 2009). Other study showed that military dogs possibly overcome these negative stimuli by developing a stronger (attachment) bond with their handlers when they use positive reinforcement training (Haverbeke, Laporte, Depiereux, Giffroy, & Diederich, 2008). Because many dog handlers do not use positive reinforcement training we cannot assume that only the dog-handler bond is enough to assure military dogs positive welfare state .(Haverbeke, Laporte, et al., 2008)

Pet dogs are a very heterogeneous group in terms of their wellbeing because their life conditions are the most diverse regarding housing, basic training, activity levels, presence or absence of other animals, of small children and ownership styles ranging from neglect/abuse to pathological attachment (Marinelli et al., 2007; Mariti et al., 2012).

The variety of lifestyles that dogs experience makes the assessment of their wellbeing challenging. Undoubtedly some dogs such as those used by the police will undergo periods of acute stress, whereas other dogs such as those in laboratories

may suffer from chronic stress. This shows that the measurement of animal welfare needs to be adjusted for every situation, for example, some measurements such as heart-rate variability might be good at detecting acute sources of stress, whereas stress hormone levels might be better at detecting longer term stress but this will depend on the biological sample used (e.g. blood gives a more instantaneous measurement than urine or faeces) (Hennessy, Davis, Williams, Mellott, & Douglas, 1997; Schatz & Palme, 2001). The idea that all stress sources should be removed from an animal's life has been debunked as a goal for the area of animal welfare. It is now acknowledged that the welfare of animals will go up and down over an individual's lifetime.

Telomeres are the sections of DNA that protect the chromosomes' ends, they naturally shorten after every cellular replication, when they become effectively eliminated this leads the cell into senescence (Aubert, 2012). Telomere shortening, or attrition can also be accelerated by poor lifestyle (e.g. low quality diet; Marcon et al., 2011; Mirabello et al., 2009; Paolisso et al., 2013) or the environment (e.g. a stressful environment; Cai et al., 2015; Gotlib et al., 2015).

In humans, the accelerated reduction of telomere length is associated with several factors such as financial struggles, smoking, depression, childhood traumas, all of them being considered as stressors (Agrigoroaei et al., 2017; Cai et al., 2015; Stephan, Sutin, & Terracciano, 2015). Recent studies with macaques, domestic dogs and birds are showing that stress is associated with telomere shortening (attrition)(Fick et al., 2012; Gardner et al., 2007; Sohn & Subramani, 2014). As stress is one of the most assessed parameters when it comes to evaluating animal welfare,

telomere attrition has now being suggested as an animal welfare measurement (Bateson, 2016). As studies have also shown that positive experiences can reduce or even reverse telomere attrition, then telomere attrition can tell us about an animal's cumulative lifetime experience; that is, whether its overall welfare level was acceptable (Bateson, 2016; Conklin et al., 2018; Jacobs et al., 2011).

Many questions regarding telomere attrition still need to be answered to allow the fully understanding of the effect of short-term stress on telomere length, in animals with different ages, to test if telomere shortening can be reversed via positive interventions, such as changes in routines or providing environmental enrichment and to replicate studies regarding telomere attrition among different mammal species to understand mechanisms and similarities (Bateson, 2016).

Studies conducted regarding telomere attrition in dogs aimed to measure the influence of telomere length on mortality, life span and telomerase (an enzyme that protects the telomeres) activity in dog tissues (Fick et al., 2012; Kraus, Pavard, & Promislow, 2013; Nasir et al., 2001; Selman, Nussey, & Monaghan, 2013). It has been found that small dog breeds have longer telomeres and therefore have a longer life span, whilst large dogs die young mainly because they age more quickly and have been found to possess shorter telomeres (Fick et al., 2012; Kraus et al., 2013). It was also found that telomeres play a critical role in determining the lifespan of somatic cells (Nasir et al., 2001). However, no study regarding dog welfare and telomere attrition has been published to our knowledge.

Quality of life is a concept to evaluate the cumulative effects of life events, both positive and negative, that impact on animal's live (Yeates, 2016). However,

there is a growing discussion about methods that could better evaluate quality of life, most of them rely on behavioural, physiological, psychological and veterinary assessments (Wojciechowska & Hewson, 2005).

The present study reports the development and validation of the use of relative Telomere Length (rTL) and its relationship with a dog's background as a (cumulative) quality of life assessment tool for use in domestic dogs (*Canis familiaris*) as a non-invasive technique.

4.2 Objective

Evaluate if the different backgrounds of dogs are associated with telomere length.

4.3 Justification

Animal welfare is influenced by the accumulative effects of pleasant and unpleasant events during an individual's life. Currently assessing welfare relies on behavioural, cognitive and physiological measurements; however, these often fail to provide an assessment of the lifetime animal welfare (i.e. if in overall did the animal have good or bad welfare (Yeates, 2016)). Using telomeres length as a tool to assess lifetime animal welfare is relatively new and no research has been conducted regarding canid welfare using telomere studies; that is, there is a significant gap in our knowledge.

4.4 Methods

4.4.1 Ethical statement

The data for this project were collected under ethical approval number: STR1617-22 conceded by The University of Salford's Ethic Committee (Appendix 1).

All owners and institutions involved were clarified about the project's objectives and received an invitation letter, information letter and a consent form (Appendices 2, 3 and 4). All biological material collected for this study is authorised under license number: ITIMP16.1096 (Appendix 5).

4.4.2 Subjects' background

Besides buccal cells samples, we also collected information regarding their background to investigate possible factors affecting the dogs' telomere length. We asked if the current owner was the first owner or if the dog was adopted, if the dog was neutered, healthy, if had any kind of training and if it received food treats. We also asked where the dog slept, how frequently it was walked and with how many people and other animals the dog had contact with (*Table 9*).

For working dogs, we also collected information about the type of work the dog was involved with (*Table 10*).

Table 9 Dogs' background information categories collected

Sex	Age	Breed Group	Origin	Neutered	Healthy	Training	Treat	Sleep	Walk	People Contact	Animal Contact
1 Male	Years	1 Gundog	1 First owner	1 No	1 Yes	1 No	1 No	1 Kennel outside	1 Everyday	1 One to two people	1 No contact
2 Female		2 Hound	2 Adopted	2 Yes	2 No	2 Yes	2 Yes	2 Inside house	2 Once a week	2 Between 3-4 people	2 One to two animals
		3 Pastoral	3 Laboratory	3 Vasectomy				3 Owners bed	3 Don't walk	3 More than 5 people	3 Between 3-4 animals
		4 Terrier							4 Every other day		4 More than 5 animals
		5 Toy									
		6 Utility									
		7 Working									
		8 Mix									

Table 10 Police dogs' working routine information collected

Working type
1 Drugs, guns
2 Tracking
3 Breeder
4 Educational dog
5 Explosives
6 Search dog
7 Retired

4.4.3 Subjects

We sampled 262 domestic dogs for telomere length assessment. Among the domestic dogs we sampled were pet dogs, shelter dogs, police dogs, laboratory dogs, rehomed dogs and behavioural research dogs. Although laboratory dogs and research dogs might appear similar these groups have very different enclosure settings and cognitive training approaches, which will be further described.

4.4.3.1 Pet dogs

Pet dogs were our biggest sample group, with 84 individuals, 45 males and 39 females, ranging from 1 to 13 years old. Our pet group was comprised of dogs from Brazil and dogs from UK. In the UK we collected dog's samples during *The Family Pet Show Manchester* 2017, this event occurs yearly lasting two days where pet owners can find pet products, see dog agility displays, training and grooming services (*Figure 12*). During the event pet owners were invited to participate in the research, and after a brief project explanation the owner decided if they were going to take part in the research and then signing the consent form (Appendix 3) (*Figure 13*). This group was the most diverse regarding breeds, with dogs from 31 different breeds. Dogs from this category were either bought or adopted and individuals had the most diverse husbandry routines between each other when considering their backgrounds.

Figure 12 Project stand on The Family Pet Show 2017 Manchester, UK where the dogs were both sampled and photographed

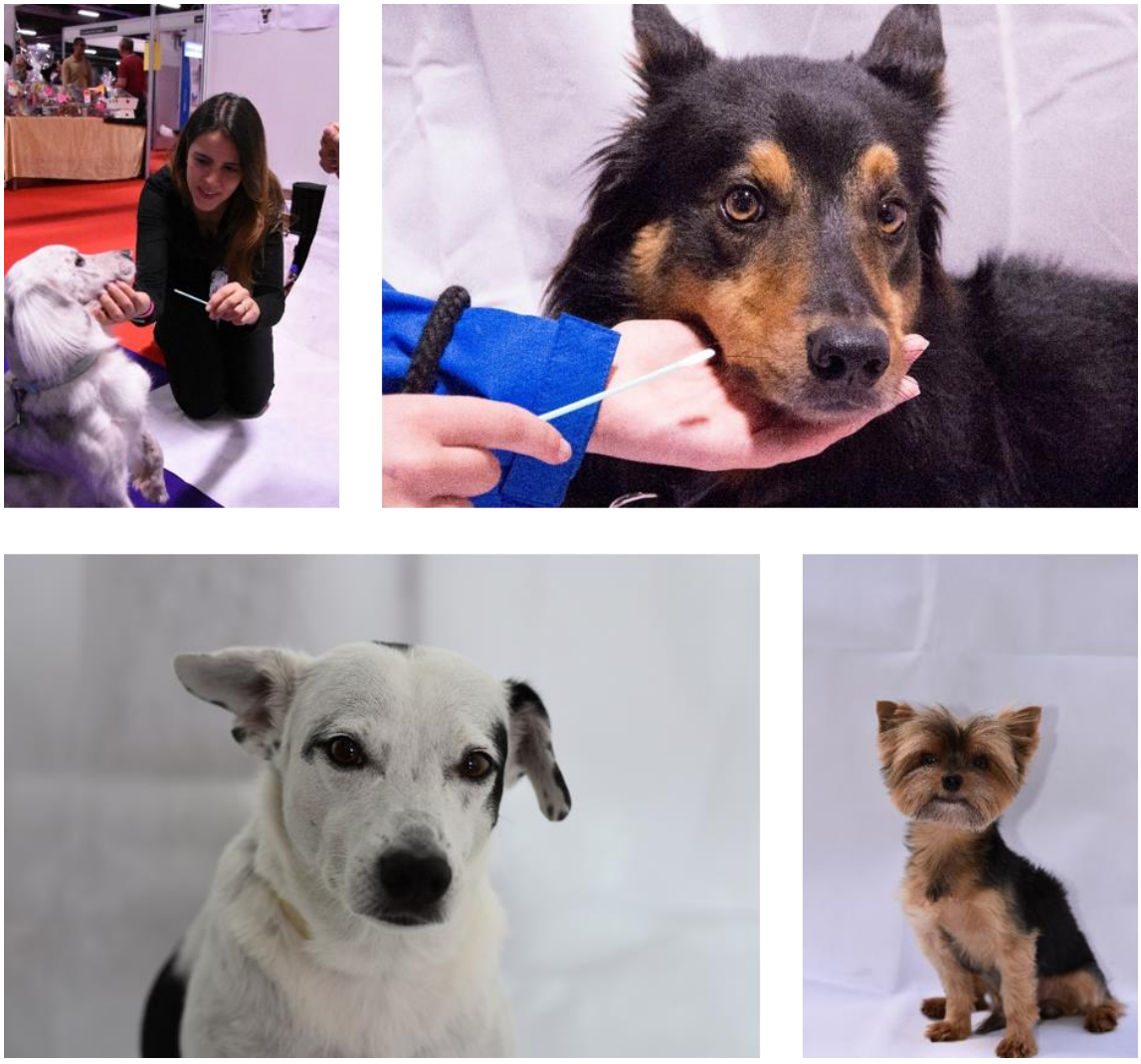

Figure 13 Pet dogs being sampled during The Family Pet Show 2017 Manchester, UK using an oral swab to collect buccal cells (i.e. DNA) and pet dogs being photographed after swab collection in Brazil

4.4.3.2 Shelter dogs

We sampled 56 dogs, 33 males and 21 females from a shelter in Brazil (Minas Gerais); their ages ranged from 2 to 10 years old. All dogs from this groups were mixed breeds; all dogs were castrated or neutered. Dogs were kept in a farm-like environment, each enclosure was around 800 m² and housed from 3 to 7 dogs. Because their housing was spacious enabling run and play activities these dogs were not walked *(Figure 14*).

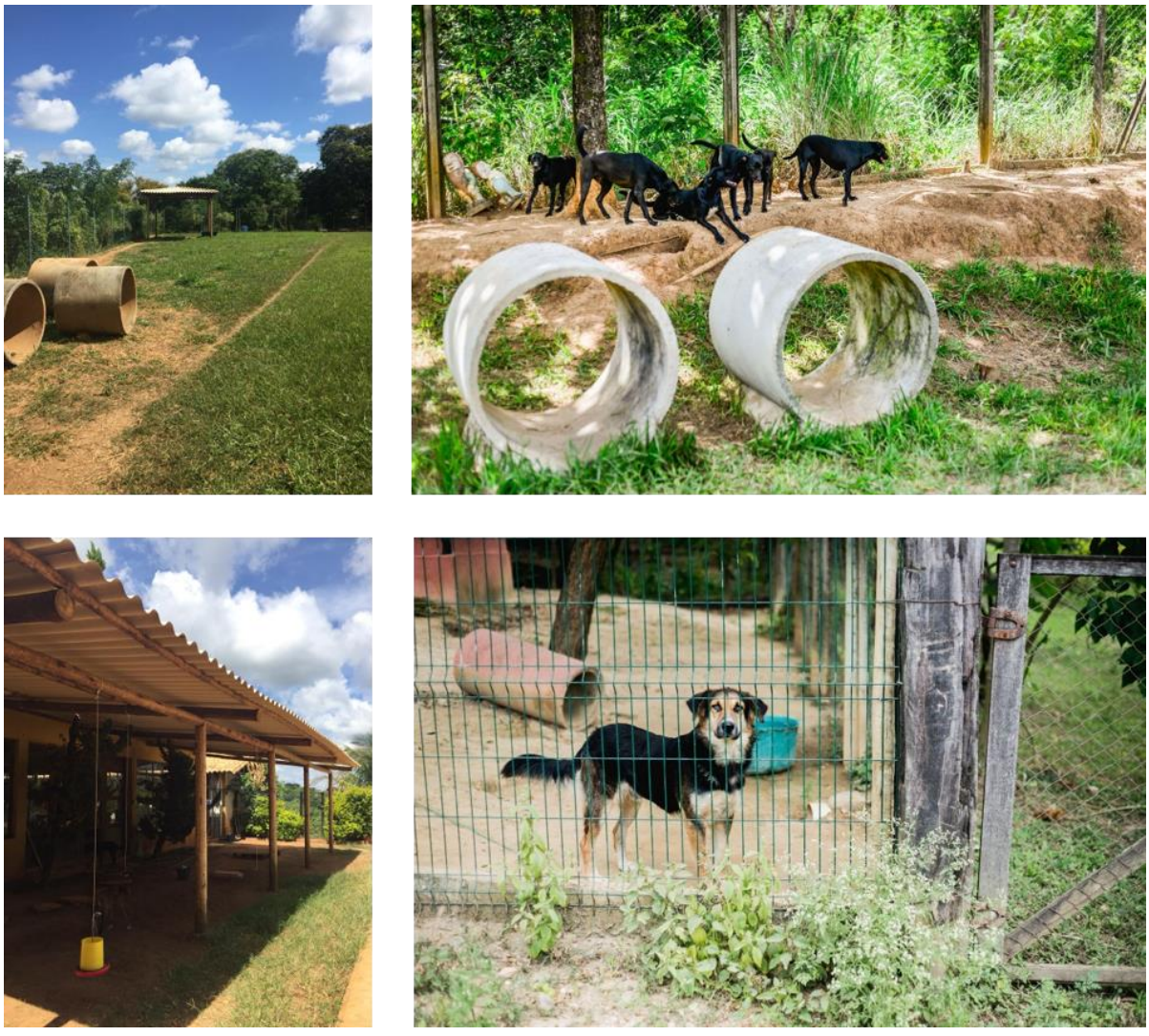

Figure 14 Photographs of dog shelter facilities in Brazil

4.4.3.3 Police Dogs

We sampled 65 police dogs, 48 males and 17 females from Brazilian and British police groups. Their ages ranged from 1 to 13 years old. Dogs from Brazil were mostly German and Belgian Shepherds, with few Labradors and Border Collies included from the Fire Department. Dogs from UK were either German Shepherds or Springer Spaniels (*Figure 15*). Brazilian dogs were kept in individual kennels (6.00m x 2.00m x 2.00m) when not working, UK dogs would go home with their police officer after working their shift. Brazilian dogs worked every other day and UK dogs worked every day. Because of the different working schedule and sleeping arrangements we divided this group into Work BR and Work UK.

Figure 15 Photographs of Police Working dogs in the UK and Brazil

4.4.3.4 Laboratory Dogs

We sampled 21 dogs, 8 males and 13 females from a laboratory environment. These dogs belong to the Veterinary School of University of Ouro Preto, Brazil. When samples were collected none of the dogs were involved in any research activity, they were all healthy, fed twice daily (09:00 and 15:00) and kept in kennels with concrete flooring, divided by walls, that were approximately (5.80m x 1.60m x 1.65m). These dogs were all mixed breeds and were not walked (*Figure 16*).

Figure 16 Laboratory facilities and dogs in Brazil

4.4.3.5 Rehoming dogs

We sampled 23 dogs, 9 males and 14 females from a rehoming facility in the UK. This facility received dogs that were not suitable for hunting activity and re-homed them. We collected samples mainly from Spaniels, Labradors and Golden Retrievers. All dogs were trained and walked at least twice a day (*Figure 17*).

Figure 17 Examples of rehoming dogs that were sampled

4.4.3.6 Behavioural Research dogs – WSC dogs

We sampled 15 dogs, 8 males and 7 females from a behavioural research centre. These dogs belong to the Wolf Science Centre (WSC) in Austria. The centre studies the commonalities of wolves, dogs and humans. Their dogs are hand-raised by scientists and are regularly taking part in cooperation and cognitive tasks just as their wolves do, which allows them to be compared in research tasks. Dogs were all mixed breed, were kept in groups of 3 up to 5 individuals, were kept in groups, in outside enclosures (4000–8000 m^2) equipped with trees, bushes, logs, and shelters (*Figure 18*). The dogs were daily walked and trained for cognitive tasks.

Figure 18 Behavioural Research dogs – WSC dogs, Austria

4.4.4 Swab collection

Swab samples were collected either by the researcher, owner, keeper or by the vet (i.e., whoever was more appropriate for the sampling and would cause least discomfort to the animal) following the steps as described in Chapter 2.

4.4.5 DNA Extraction

In December 2016 a pilot study was undertaken to determine the best method for extracting DNA from the swabs. DNA was extracted using the Isohelix Buccalyse DNA Release Kit (Cell Projects, Kent, UK) and the DNeasy® Blood & Tissue kit (QIAGEN) to determine which extraction kit was going to be used for sample extractions.

4.4.6 DNA Quantification

Concentration of DNA extracted from swab samples was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Marietta, OH, USA). All samples were diluted to a working stock concentration of 50 ng/µL in PCR-grade water and single-use aliquots were kept frozen at –30 ºC.

4.4.7 qPCR assay

Each qPCR batch (i.e. run) consisted of two components: i) a tenfold point serial dilution prepared from a pool of DNA from 264 dogs from 100.7 ng/µl, which was used to construct the standard curve and formed the basis of each primer optimization and quality control; ii) no template controls (NTC), which allowed for detection of potential contamination and/or primer dimer formation. This was done for the telo-

mere gene and the reference copy gene. Each step of the dilution series and the NTC were run in triplicate in each batch (Olsen, Bérubé, Robbins, & Palsbøll, 2012).

The primers, qPCR reaction mix and conditions were described previously in Chapter 2.

4.4.8 Measuring relative telomere length (RTL)

The measurement of the relative telomere length was calculated by using an adaptation of the qPCR method described by Cawthon (2002), as described previously in Chapter 2.

4.4.9 Data collection and statistical analyses

Dog breeds were classified in accordance with the UK's The Kennel Club (KC) criteria, organization founded in 1873, originally focused on pure-bred dogs activities and currently promoting responsible dog ownership (KC, www.thekennelclub.org.uk) (The Kennel Club, 2019). The Kennel Club classification, worldwide recognized, is based on the breed historical function and as almost half of our sampling was from mixed breed dogs we created and added the "Mix" category. The 39 breeds present in this study were therefore classified into eight groups (*Table 11*).

Table 11 Dog breed groups in the present study according to The Kennel Club (UK)

Breed Group	Description	Number of individuals
Gundogs	Dogs that were originally trained to find live game and/or to retrieve game that had been shot and wounded.	46
Hounds	Breeds originally used for hunting either by scent or by sight	5
Pastorals	The Pastoral Group consists of herding dogs that are associated with working cattle, sheep, reindeer and other cloven-footed animals.	59
Terriers	Dogs originally bred and used for hunting vermin.	5
Toys	The Toy breeds are small companion or lap dogs.	23
Utility	This group consists of miscellaneous breeds of dog mainly of a non-sporting origin, including the Bulldog, Dalmatian, Akita and Poodle	20
Working	Over the centuries these dogs were selectively bred to become guards and search and rescue dogs.	3
Mix	Mixed breed dogs	103

According to their background's dogs were also placed into seven different groups (*Table 12*)

Table 12 Dogs group categories based on their backgrounds

Group	Description	Number of individuals
BR Work	Police dogs from Brazil	45
Lab	Dogs from a veterinary laboratory facility	21
Pet	Pet dogs from UK and Brazil	84
Rehome	Dogs from a training facility that were going to be soon re-homed	23
Shelter	Dogs from a shelter in Brazil	54
UK Work	Police dogs from UK	20
WSC	Dogs from a behavioural research centre	15

To confirm if the dogs' grouping based on their backgrounds were adequate for further analysis a linear discriminant analysis was performed. The purpose of dis-

criminant analysis is to discriminate between two or more naturally occurring groups based on a suite of continuous or discriminating variables (Deakin, 1972). The analysis allows us to determine underlying, dominant gradients of variation between groups of samples and can be used not only to explain differences among groups but also to predict group membership for samples from unknown groups (Fraley & Raftery, 2002). For the first performed discriminant analysis, the predictor was the relative telomere length and the clustering group was the dog's background group. The second discriminant analysis had relative telomere length as the predictor and the dogs age as the clustering group.

The relative Telomere Length (rTL) was the response factor for all models and as the residuals of rTL were normally distributed a Generalised Linear Model was used to identify the background factors that had an influence on dogs' rTL. To obtain the optimal model the function drop1 was used to remove all non-significant variables, all models were then checked using the Akaike's information criterion (AIC) and then the most explanatory model was chosen.

In the first model we selected dogs from the same age, the two years old because they were the largest sample and investigated if their relative telomere length were affected by sex, breed, group category, origin, neutered, health, training, food treats, sleep, walk, and animal contact. Sixty-four dogs were selected for this analysis.

In the second model we investigated if a dog's size would impact the relative telomere length, so we selected all pure-breed dogs and used breed's size chart from The Kennel Club (KC, www.thekennelclub.org.uk) (The Kennel Club, 2019).

Size was added to the factors sex, age, breed group, origin, neutered, health, training, food treats, sleep, walk, people contact, and animal contact to evaluate if they had any influence on dogs' rTL. A hundred and forty-nine dogs were selected for this analysis.

In the third model we investigated if a dog's sex, age, breed group, origin, neutered status, health, training, food treats, sleep location, walk activity, people contact, and animal contact had any influence on dogs' rTL. Two hundred sixty-two dogs were selected for this analysis.

The fourth model examined if the country, Brazil or UK, the working schedule and working type had any influence on the relative Telomere Length of police dogs. All data were tested for normality (Kolmogorov–Smirnov Test) and the results of all statistical tests were considered significant at $p < 0.05$. Sixty-five dogs were selected for this analysis.

All statistical analysis were run under the platform Studio R (Dworschak, Koller, & Abed-Navandi, 2006) and Minitab (Minitab Inc., 2010).

4.5 Results

4.5.1 DNA extraction kits - Pilot Study

DNA from six swab samples was extracted using the Isohelix Buccalyse DNA Release Kit (Cell Projects, Kent, UK) and another six swab samples were extracted using the DNeasy® Blood & Tissue (QIAGEN). After extraction, the DNA was quantified using the Qubit™ 3.0 Fluorometer and sensitive, specific Qubit™ quantitation assays. From the Buccalyse DNA Release Kit DNA concentration ranged from 0.76

to 8.91 ng/µL and from 1.32 to 9.58 ng/µL using the DNeasy® Blood & Tissue (QIAGEN). A T-test showed that there was no significant difference between the DNA yield obtained from the two kits ($t(7) = 0.82$, $p > 0.05$). For this reason, we chose to work with Buccalyse DNA Release Kit because it was simpler and more cost effective.

4.5.2 Grouping Dogs

To evaluate if the dog's groups based on their background were accurate a discriminant analysis was performed using rTL as a predictor and the different dogs' groups were de dependent variable for grouping the dogs. The outcomes showed that the accuracy of the dogs' groups was 94%, which indicated that further analysis could be done with the common background grouping originally established (*Table 13*).

Table 13 Discriminant analysis for dogs' groups

	True Group						
Group allocation	BRWork	Lab	Pet	Rehome	Shelter	UKWork	WSC
BRWork	45	0	1	0	0	0	0
Lab	0	21	0	0	0	0	0
Pet	0	0	72	0	0	0	0
Rehome	0	0	3	23	0	0	0
Shelter	0	0	1	0	54	0	0
UKWork	0	0	5	0	0	20	0
WSC	0	0	2	0	0	0	15
Total N	45	21	84	23	54	20	15
N correct	45	21	72	23	54	20	15
Proportion	1.000	1.000	0.857	1.000	1.000	1.000	1.000

4.5.3 Relative Telomere Length and Age in dogs' groups

The groups Pet and UKWork had the biggest age range while dogs from the Rehome group had the smallest age range. Dogs from Lab had the shortest rTL mean between the groups while dogs from the behavioural research centre (WSC) had the longest rTL mean. Results are summarized in Table 14.

Table 14 rTL and age mean for each dog's background group

Category	rTL Mean	rTL Range	Age Mean (years)	Age Range (years)	N
Lab	0.68	0.61 – 0.74	3.52	1 – 8	21
Pet	0.71	0.55 – 0.90	4.25	1 – 13	84
Rehome	0.75	0.66 – 0.94	2.00	1 – 5	23
Shelter	0.75	0.54 – 0.98	4.00	2 – 10	54
UK Work	0.77	0.57 – 0.96	5.30	1 – 14	20
BR Work	0.78	0.63 – 0.97	3.31	1 – 8	45
WSC	0.81	0.67 – 0.91	4.86	2 – 8	15

4.5.4 Association of rTL and Age

To evaluate if the dogs would be properly grouped by age a discriminant analysis was performed using rTL as a predictor for grouping the dogs by their ages. The outcomes showed that the accuracy of the dogs' age groupings was 27.5%, dogs from young age were more frequently wrong assigned and old dogs in general were more correctly assigned to their true ages (Table 15 and Table 16). Discriminant analysis required at least two samples from each category, so one 14-year-old dog from UK Work group was removed for this analysis.

When considering the rTL mean across all dog ages sampled it was noticeable that the rTL mean varied less in younger dogs (Table 16) (*Figure 19*).

Table 15 Discriminant analysis for dogs' ages groups using relative telomere length as the predictor

	True Group												
Put into Group	1	2	3	4	5	6	7	8	9	10	11	12	13
1	12	8	3	1	1	0	3	0	0	0	1	0	0
2	3	17	3	0	1	2	1	0	0	0	0	0	0
3	3	5	7	3	3	2	0	0	0	0	0	0	0
4	1	3	2	5	1	0	1	0	0	0	0	0	0
5	7	14	9	2	9	5	2	0	1	0	0	0	0
6	8	1	2	0	3	7	0	1	0	0	0	0	0
7	0	0	1	0	0	0	1	0	0	0	0	0	0
8	8	9	1	0	0	1	0	2	0	0	0	0	0
9	0	1	2	4	3	1	4	0	5	1	0	0	0
10	0	0	0	2	0	0	1	1	0	0	1	0	0
11	1	3	1	0	0	1	2	0	0	2	2	0	0
12	7	2	2	3	0	1	3	0	0	1	0	3	0
13	1	1	4	0	1	1	2	1	0	1	1	0	2
Total N	51	64	37	20	22	21	20	5	6	5	5	3	2
N correct	12	17	7	5	9	7	1	2	5	0	2	3	2
Proportion	0.250	0.266	0.189	0.250	0.409	0.333	0.050	0.400	0.833	0.000	0.400	1.000	1.000

Table 16 Relative telomere length by each dog's age sampled

Age	Total Count	Mean	SE Mean	Median	IQR
1	51	0.7461	0.0123	0.7190	0.1165
2	64	0.7497	0.0102	0.7435	0.0988
3	37	0.7618	0.0131	0.7491	0.1054
4	20	0.7272	0.0142	0.7247	0.0854
5	22	0.7523	0.0152	0.7382	0.0969
6	21	0.7509	0.0176	0.7442	0.1374
7	20	0.7419	0.0155	0.7416	0.0717
8	5	0.8005	0.0497	0.8038	0.1903
9	6	0.7516	0.0209	0.7482	0.0886
10	5	0.7447	0.0304	0.7230	0.1297
11	5	0.6723	0.0418	0.6589	0.1796
12	3	0.6737	0.0536	0.7001	0.1800
13	2	0.792	0.1460	0.7920	*
14	1	0.76000	*	0.7600	*

IQR = interquartile range

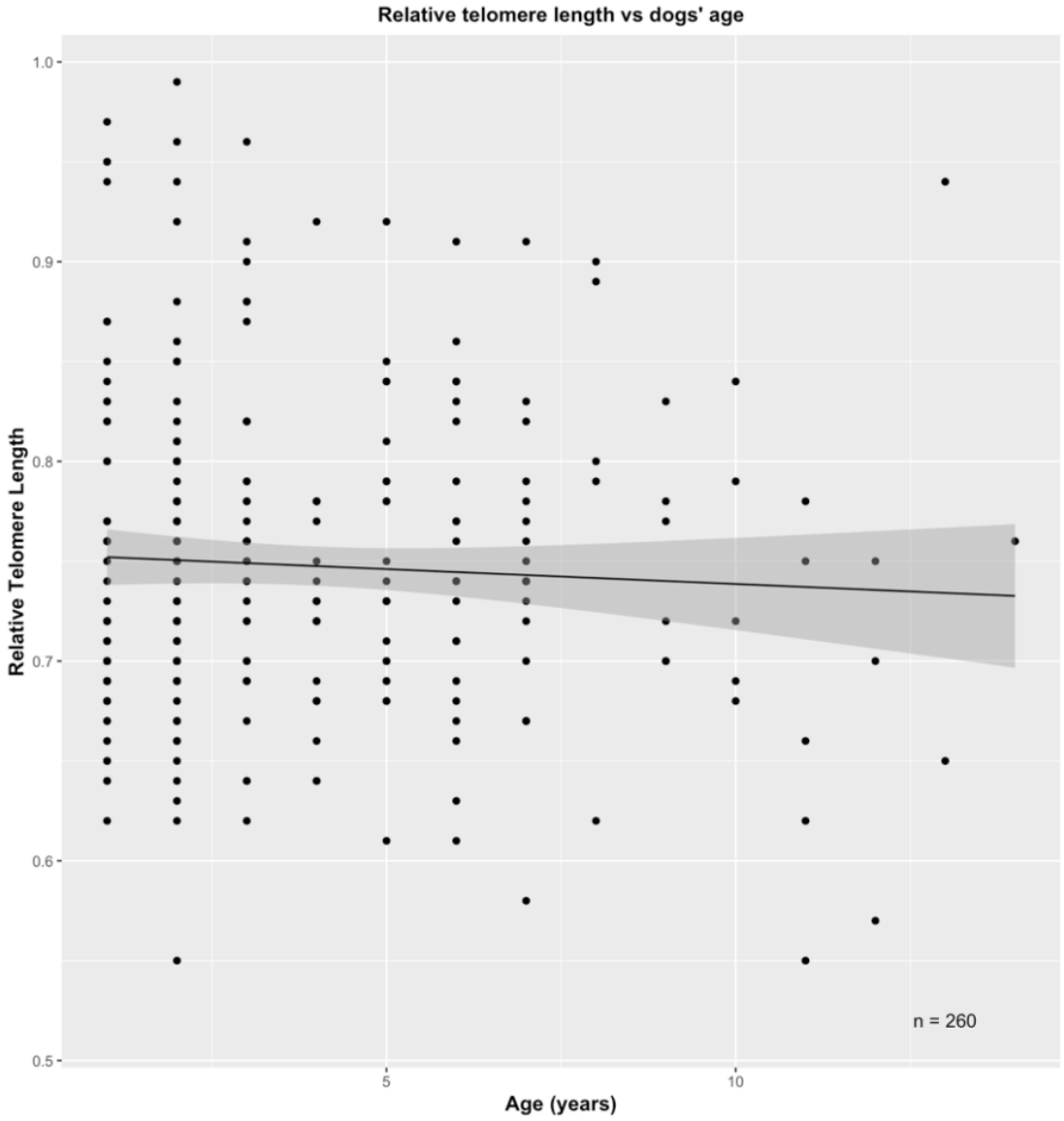

Figure 19 Relative Telomere Length by dog's age

4.5.5 Model 1: Association of rTL and two-year-old dogs

The residuals of relative Telomere Length were normally distributed, so the first GLM model was performed with Gaussian distribution including all background factors. We used the function drop1 to remove all non-significant variables for the optimal model that was achieved with only the factor category group showing a significant impact on the relative telomere length (R^2= 0.282, $p < 0.05$) (*Table 17*) (*Figure 20*).

Table 17 Final Generalised linear model result for the effects of group category on relative telomere length of two years old domestic dogs [1]

Parameters	Estimate±SD	t value	P
Intercept	0.8175 ± 0.0257	31.709	<0.0001***
CategLab [ab]	-0.1255±0.0415	-.3.019	0.0038**
CategPet	-0.1159 ±0.0307	-3.772	0.0004***
CategRehome	--0.0593±0.0338	-1.751	0.0854
CategShelter	-0.0359±0.0307	-1.169	0.2474
CategUKWork	-0.1075 ±0.0773	-1.390	0.1700
CategWSC	-0.03750±0.0774	-0.485	0.6296

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -144.94

[a] Explanatory variables included in the full model: sex, breed, group category, origin, neutered, health, training, food treats, sleep, walk, and animal contact

[b] Laboratory, Pet, Rehome, Shelter, UK Work, BR Work, WSC: BR Work is the reference group

• P ≤ 0.05

* P ≤ 0.01

** P ≤ 0.001

*** P ≤ 0.0001

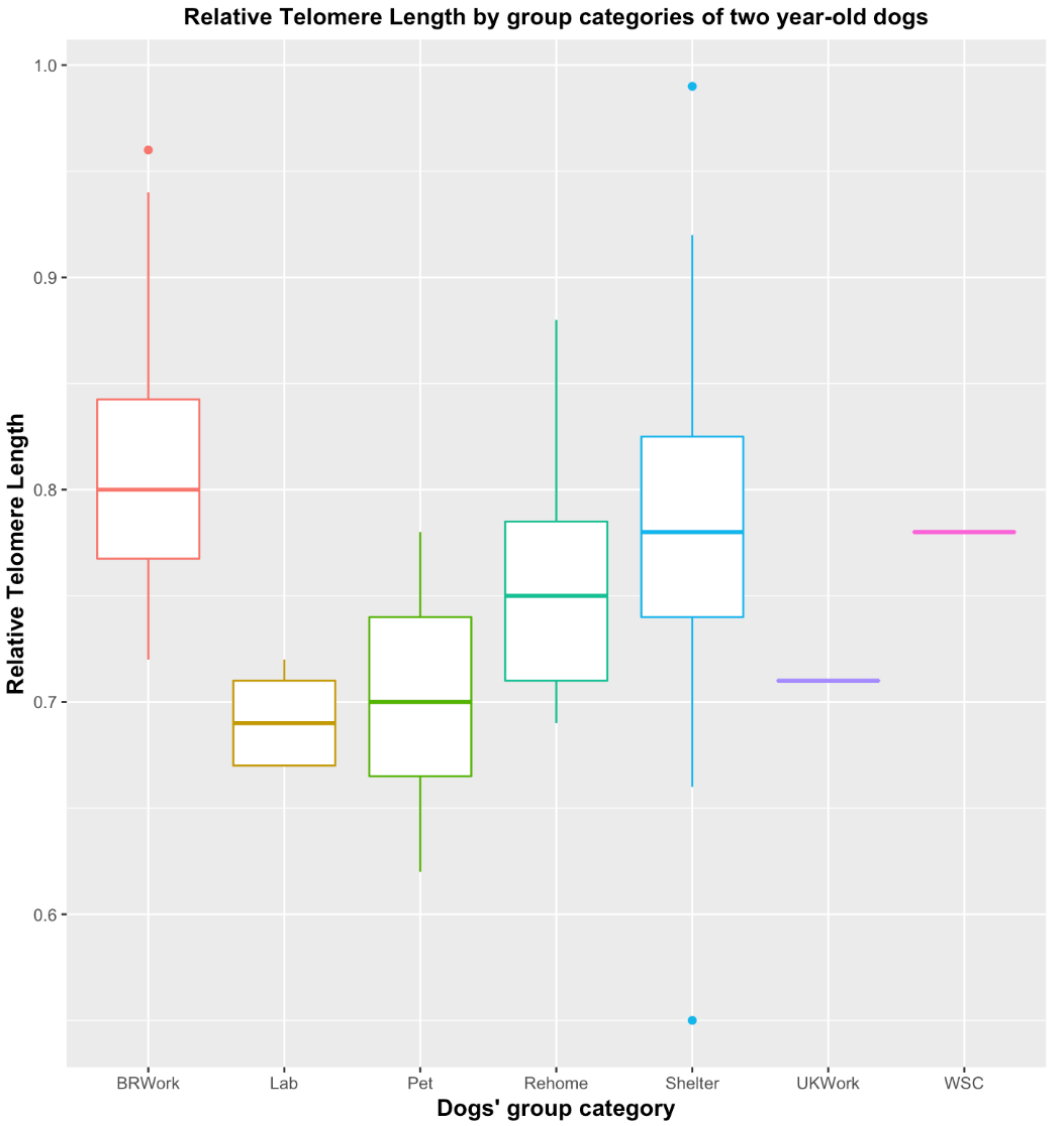

Figure 20 relative Telomere Length by group categories of two-year-old dogs

4.5.6 Model 2: Association of rTL and dogs' breed

In the second model we selected pure breed dogs and evaluated if their relative Telomere Length was affected by sex, size breed, group category, origin, neutered, health, training, treats, sleep, walk, and animal contact.

The residuals of relative Telomere Length were normally distributed, so the first GLM model was performed with Gaussian distribution including all background factors and using the function drop1 to remove all non-significant variables for the optimal model achieved with the factors category group, sex, food treat, sleep, walk, had a significant impact on the relative telomere length (R^2= 0.282, $p < 0.05$) (*Table 18*).

Table 18 Final Generalised linear model result for the effects of group category, sex, food treat, sleep and walk on relative telomere length dogs from different body sizes [1]

Parameters	Estimate±SD	t value	P
Intercept	0.6925± 0.0188	36.714	< 0.0001 ***
Categ: Pet [ab]	-0.2003± 0.0835	-2.397	0.0179 *
Categ: Rehome	0.0478± 0.0232	2.057	0.0416 *
Categ: UKWork	-0.1075± 0.0791	-1.359	0.1764
Sex Male [c]	0.0381± 0.0126	3.013	0.0031 **
Treat No [d]	0.1482±0.0758	1.954	0.0527
Sleep: Inside house [e]	0.064229±0.0276	2.324	0.0216 *
Sleep: Owners Bed	0.0507± 0.0280	1.809	0.0727
Walk: Once a week [f]	-0.0021±0.0287	-0.074	0.9408
Walk: Don't walk	0.07551±0.0253	2.980	0.0034 **
Walk: Every other day	0.1064±0.0224	4.746	<0.0001 ***

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -353.6739

[a] Explanatory variables included in the full model: sex, breed, group category, origin, neutered, health, training, food treats, sleep, walk, and animal contact

[b] Pet, Rehome, UK Work, BR Work: BR Work is the reference group

[c] Male or Female: Female is the reference group

[d] Food Treat Yes or Food Treat No: Food Treat Yes is the reference group

[e] Outside, Inside house, Owners' bed: Inside house is the reference group

[f] Every day, Once a week, Don't walk, Every other day: Every day is the reference group

• P ≤ 0.05

* P ≤ 0.01

** P ≤ 0.001

*** P ≤ 0.0001

4.5.7 Model 3: Factors that impact on a dog's relative Telomere Length

The residuals of relative Telomere Length were normally distributed, so the third GLM model was performed with Gaussian distribution including all background factors and using the function drop1 to remove all non-significant variables achieved the optimal model where the factors category group, sex, sleep, walk and animal contact had significant impact on the relative telomere length (R^2= 0.326, $p < 0.05$). Although the significance of the factor sex can be disputed ($t = 1.96$, $df = 1$, $p = 0.052$) it was maintained in the current model, since an alternative model was tested removing the factor sex, but the AIC indicated that most explanatory model was Model 1, the one that included the factor sex. GLM results for Model 2 are summarized in Table 19.

Table 19 Final Generalised linear model result for the effects group category, sex, sleep, walk, and animal contact on relative telomere length of domestic dogs [1]

Parameters	Estimate±SD	t value	P
Intercept	0.8430 ±0.0471	17.867	< 0.0001 ***
Categ Lab [ab]	-0.1327± 0.0345	-3.843	0.0001 ***
Categ Rehome	-0.0105±0.0280	-0.378	0.7058
Categ Shelter	-0.0276±0.0274	-1.005	0.3159
Categ UKWork	-0.0304±0.0376	-0.808	0.4196
Categ WSC	0.0365±0.0309	1.181	0.2388
Sex Male [c]	0.0177±0.0090	1.962	0.0590
Sleep Kennel outside [d]	-0.0790±0.0315	-2.503	0.0129 *
Sleep Owners Bed	0.0036 ±0.016	0.225	0.8220
Walk Every day [e]	-0.0556±0.0228	-2.438	0.0154 *
Walk Every other day	0.0510±0.0283	1.800	0.0731
Walk Once a week	-0.0800±0.0333	-2.399	0.0171 *
Animal.Contact 3-4 animals [f]	0.0177±0.0141	1.255	0.2107
Animal.Contact More than 5 animals	0.0496±0.0175	2.826	0.0051 **
Animal.Contact No contact	-0.0017±0.0176	-0.099	0.9212

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -644.40

[a] Explanatory variables included in the full model: sex, breed, group category, origin, neutered, health, training, food treats, sleep, walk, and animal contact

[b] Laboratory, Pet, Rehome, Shelter, UK Work, BR Work, WSC: BR Work is the reference group

[c] Male or Female: Female is the reference group

[d] Outside, Inside house, Owners' bed: Inside house is the reference group

[e] Every day, Once a week, Don't walk, Every other day: Every other day is the reference group

[f] No contact, 1-2 animals, 3-4 animals, More than 5 animals: 1-2 animals is the reference group

• P ≤ 0.05

* P ≤ 0.01

** P ≤ 0.001

4.5.8 Investigating significant factors from Model 3

Dogs from the laboratory group ($t = -4.93$, $df = 6$, $p < 0.05$) presented the lowest mean for relative telomere lengths and dogs from Work group, regardless if they were from Brazil ($t = 1.74$, $df = 6$, $p < 0.05$) or UK ($t = 0.33$, $df = 6$, $p < 0.05$) presented a higher mean for relative telomere length (*Figure 21*). Males presented longer relative Telomere Length when compared to females ($t = -1.96$, $df = 1$, $p < 0.05$) (*Figure 22*). Dogs that slept in a kennel outside ($t = -2.64$, $df = 2$, $p < 0.05$) showed a longer relative Telomere Length, but when allowed inside the house dogs who slept with their owners in bed had longer relative Telomere Length than the other dogs who were inside but not allowed in their owner's bed ($t = 1.94$, $df = 2$, $p < 0.05$) (*Figure 23*). Dogs who were walked every day ($t = -3.08$, $df = 3$, $p < 0.05$) or once a week had shorter relative telomere length when compared to dogs that were not walked ($t = 1.18$, $df = 3$, $p < 0.05$) or walked every other day ($t = 4.25$, $df = 3$, $p < 0.05$) (*Figure 24*). Dogs who had contact with more than five animals ($t = 2.69$, $df = 3$, $p < 0.05$) had significantly shorter relative Telomere Length when compared with dogs that had no contact with other animals (*Figure 25*).

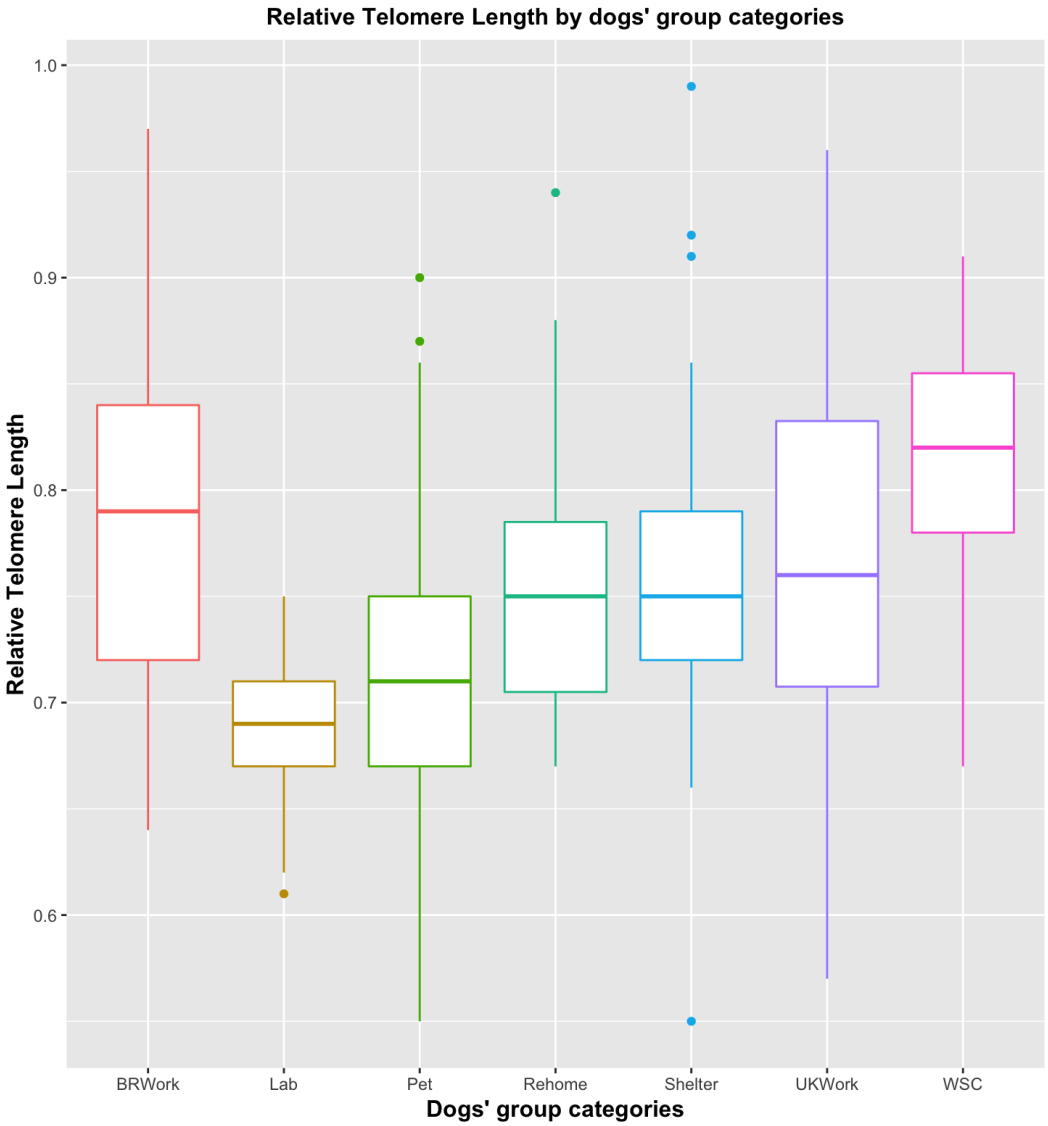

Figure 21 relative Telomere Length by dogs' categories

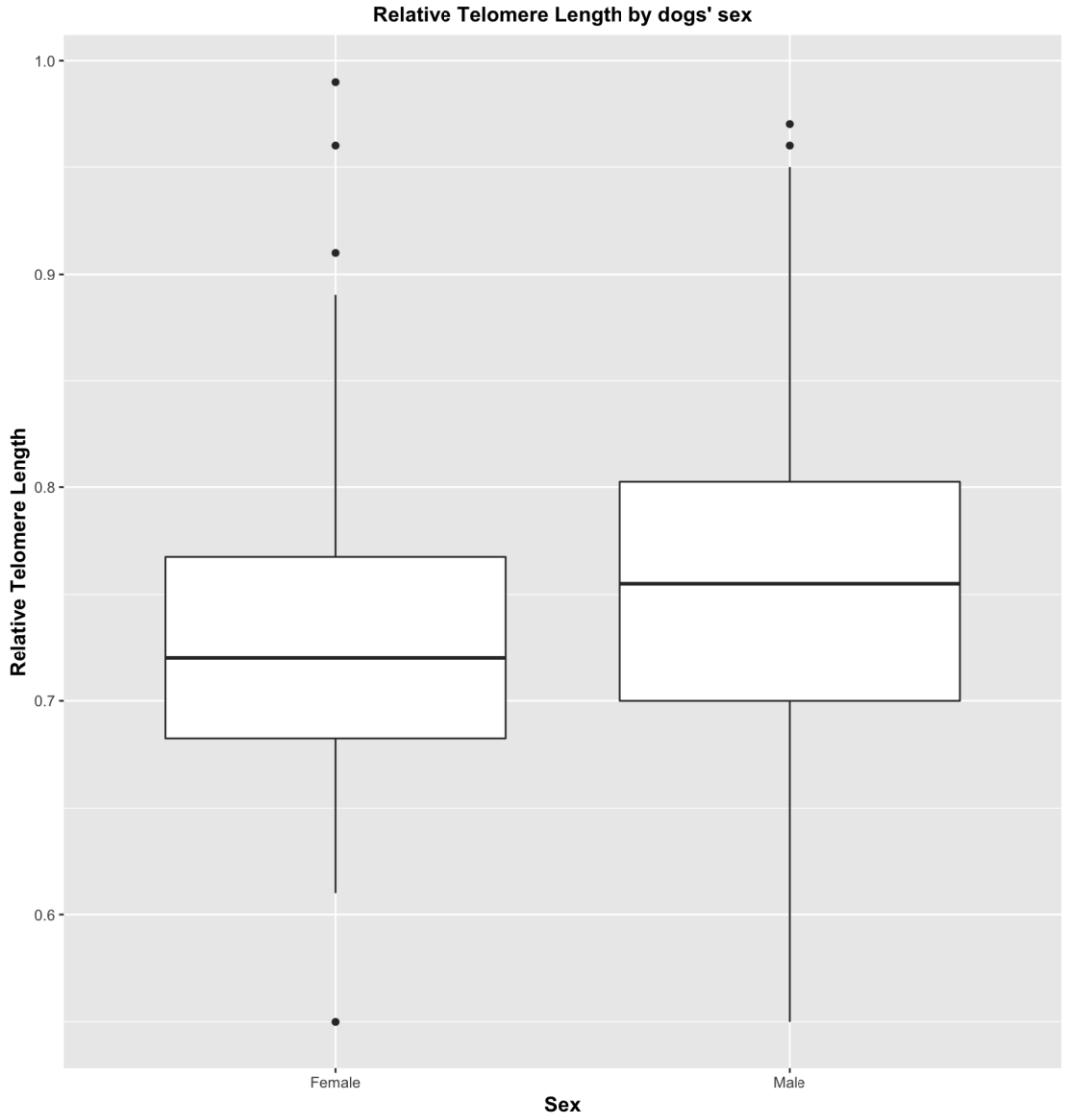

Figure 22 relative Telomere Length by dogs' sex

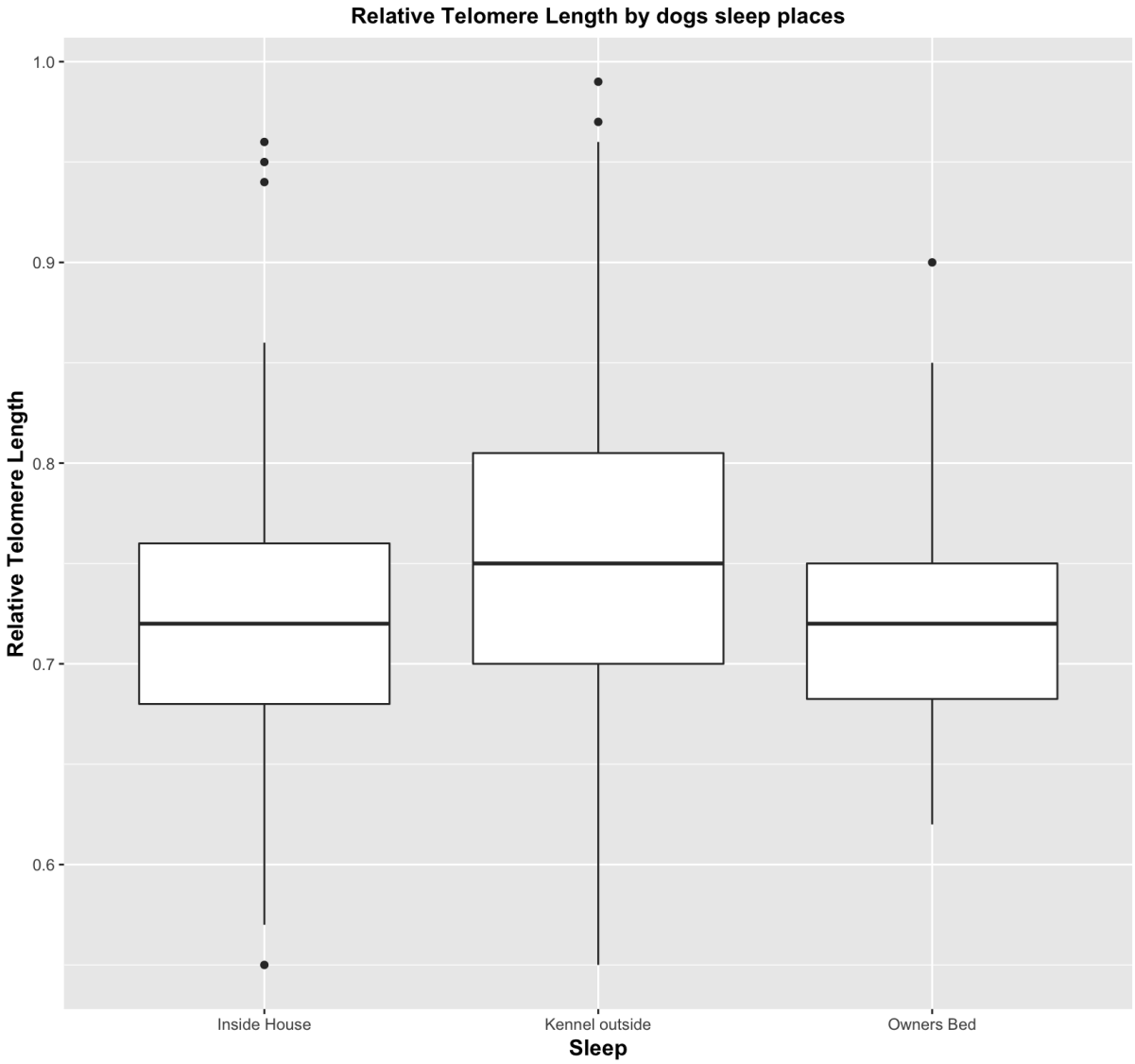

Figure 23 relative Telomere Length by dogs' sleeping place

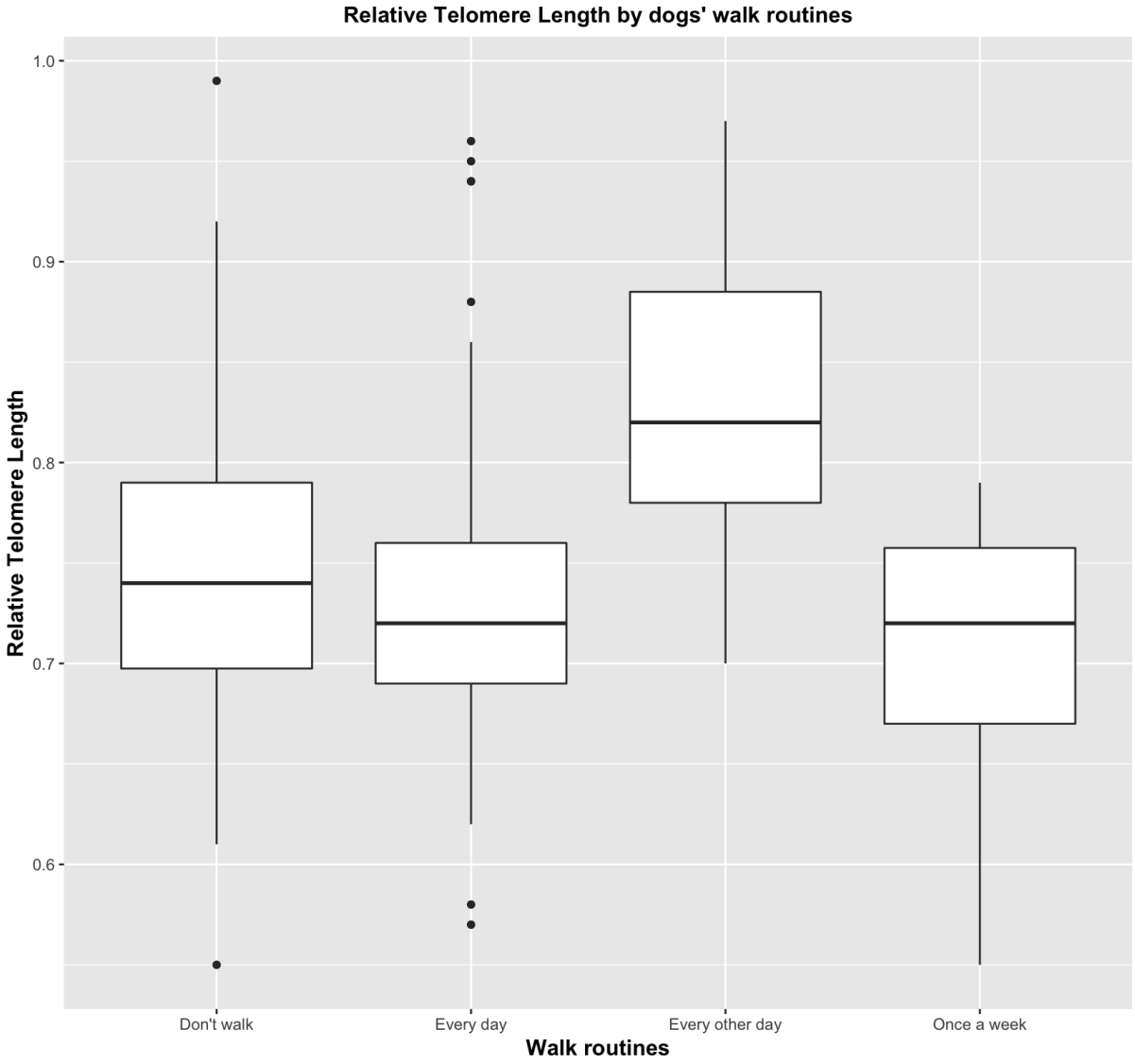

Figure 24 relative Telomere Length by dogs' walk routine

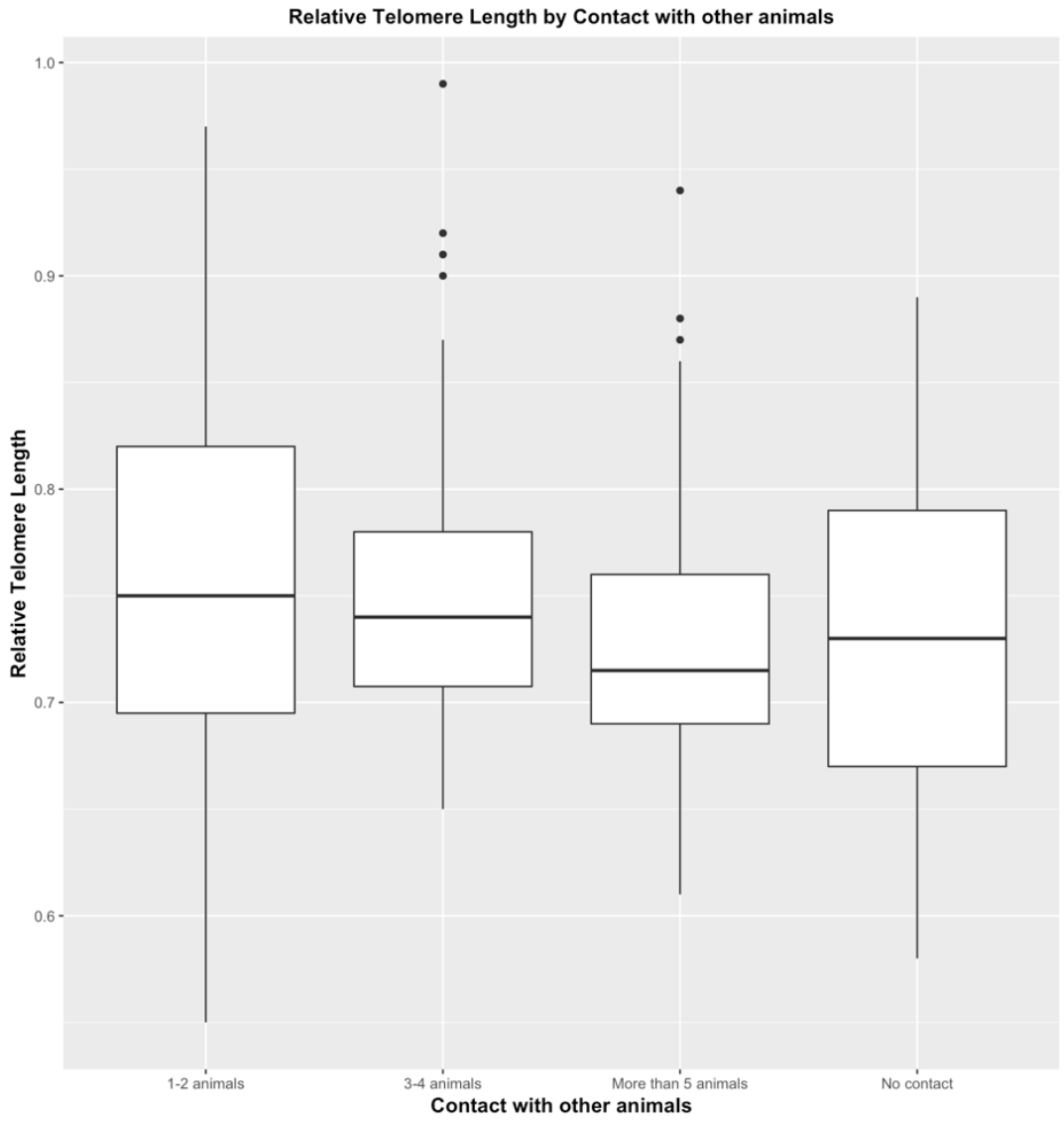

Figure 25 relative Telomere Length by dogs' contact with other animals

4.5.9 Model 4: Factors that impact on working dogs' relative Telomere Length

Since Model 2 indicated significant difference between dogs' group a further investigation was conducted to investigate possible differences between working dogs from Brazil and working dogs from UK. The dogs from these groups had the same occupation, but Brazilian police dogs and British police dogs had a few differ-

ences between them. Walk routine, sleep place, treats, and training style were consistent within the individuals of the same group, but different from one group to another. For this reason, these prediction factors were excluded from the model to avoid collinearity with the variable group. The residuals of relative Telomere Length from working dogs' group were normally distributed, hence the second GLM model was performed with Gaussian distribution including all background factors and using the function drop1 was used to remove all non-significant variables, for the achieved optimal model. Model 3 showed the factors (category group and working type had significant impact on the relative telomere length (R^2= 0.380, $p < 0.05$). Similar to what occurred in Model 1, the factor sex was not significant in Model 2 (t = 1.65, df = 1, p = 0.104). However, sex was maintained in the current Model 2, because when an alternative model was tested removing the factor sex, the AIC indicated that most explanatory model was the model that included the factor sex. GLM results for Model 3 are summarized in Table 20.

Table 20 Final Generalised linear model result for the effects group category, sex and working type on relative telomere length of working dogs [1]

Parameters	Estimate±SD	t value	P
Intercept	0.6400±0.0784	8.156	<0.0001 ***
Group UKWork [ab]	-0.0511±0.0262	-1.950	0.0561
Sex Male [c]	0.0381±0.0234	1.627	0.1093
Working.type Capture [d]	0.1730±0.0833	2.076	0.0425 *
Working.type Drugs & Guns	0.1606±0.0829	1.936	0.0579
Working.type Educational	0.0412±0.0861	0.478	0.6343
Working.type Explosives	0.1220±0.0850	1.435	0.1569
Working.type Retired	0.1506±0.0883	1.704	0.0939
Working.type Search & Rescue	0.0342±0.0855	0.400	0.6905

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Relative telomere length AICc= -644.40

[a] Explanatory variables included in the full model: sex, breed, category group, neutered, health and working type

[b] UK Work or BR Work: BR Work is the reference group

[c] Male or Female: Female is the reference group

[d] Working.type Breeder, Working.type Capture, Working.type Drugs & Guns, Working.type Educational, Working.type Explosives, Working.type Retired, Working.type Search & Rescue: Breeder is the reference group

• P ≤ 0.05

* P ≤ 0.01

** P ≤ 0.001

*** P ≤ 0.0001

4.5.10 Investigating significant factors from Model 4

Police dogs from Brazil (t = 1.99, df = 1, p = 0.05) presented a higher mean for relative Telomere Lengths than police dogs from UK (*Figure 26*). Dogs that work with Tracking (t = 3.49, df = 61, p< 0.05) and Drugs & Guns (t = 2.64, df = 61, p< 0.05) had longer relative Telomere Lengths than dogs who performed other work types (*Figure 27*). Although not statistically significant, the factor sex showed the same tendency of Model 1 where males presented longer relative telomere length when compared to females (t = 1.65, df = 1, p= 0.104) (*Figure 28*).

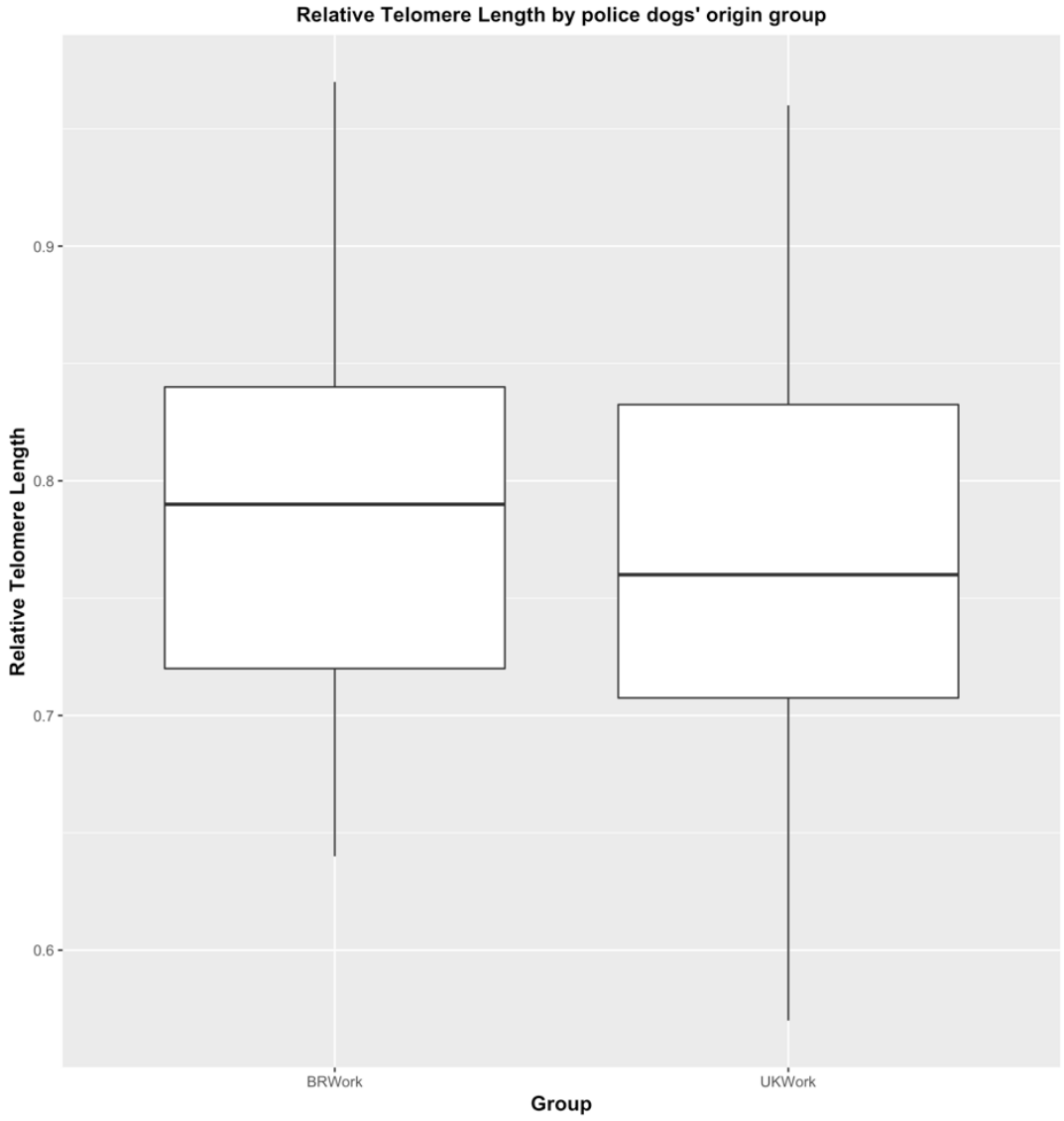

Figure 26 relative Telomere Length by police dogs' origin group

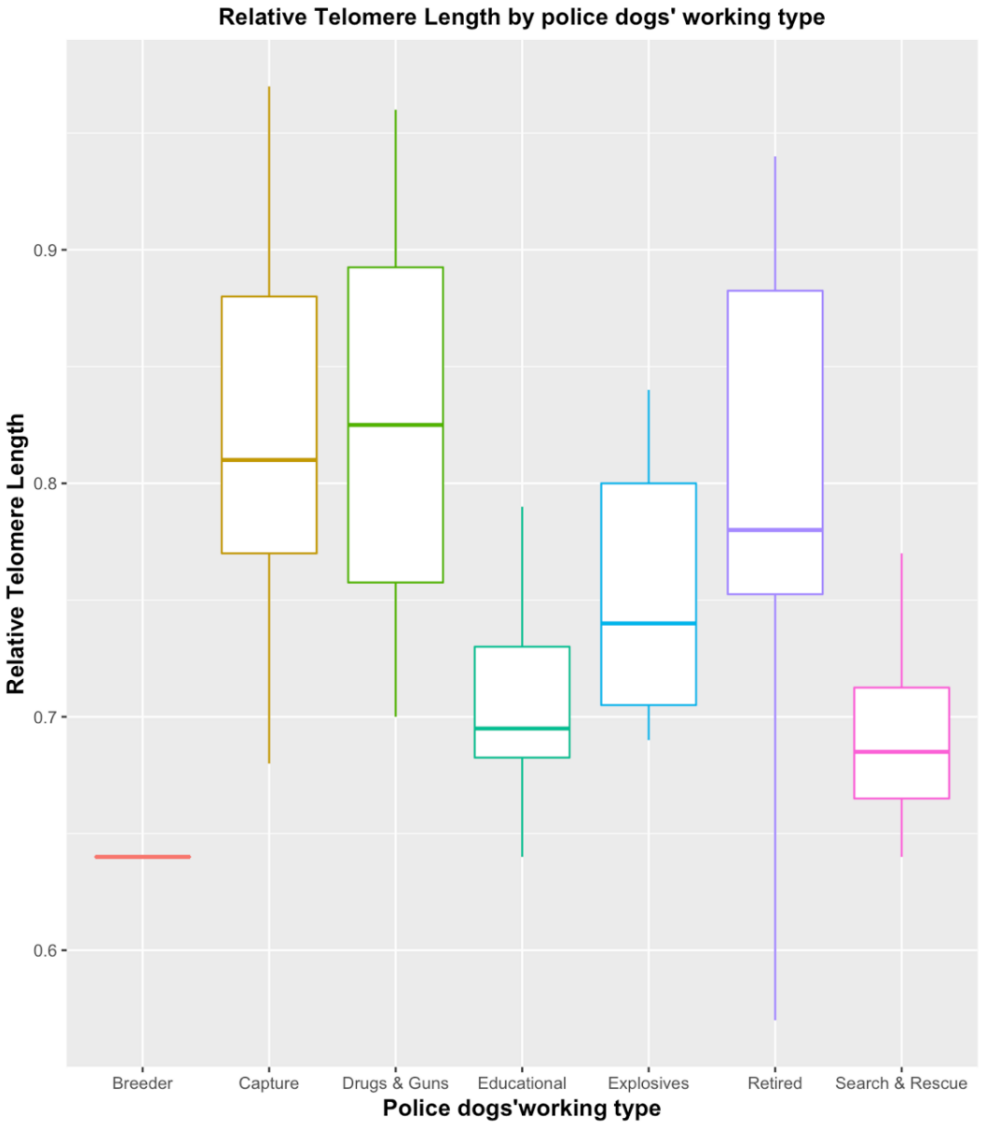

Figure 27 relative Telomere Length by police dogs' working type

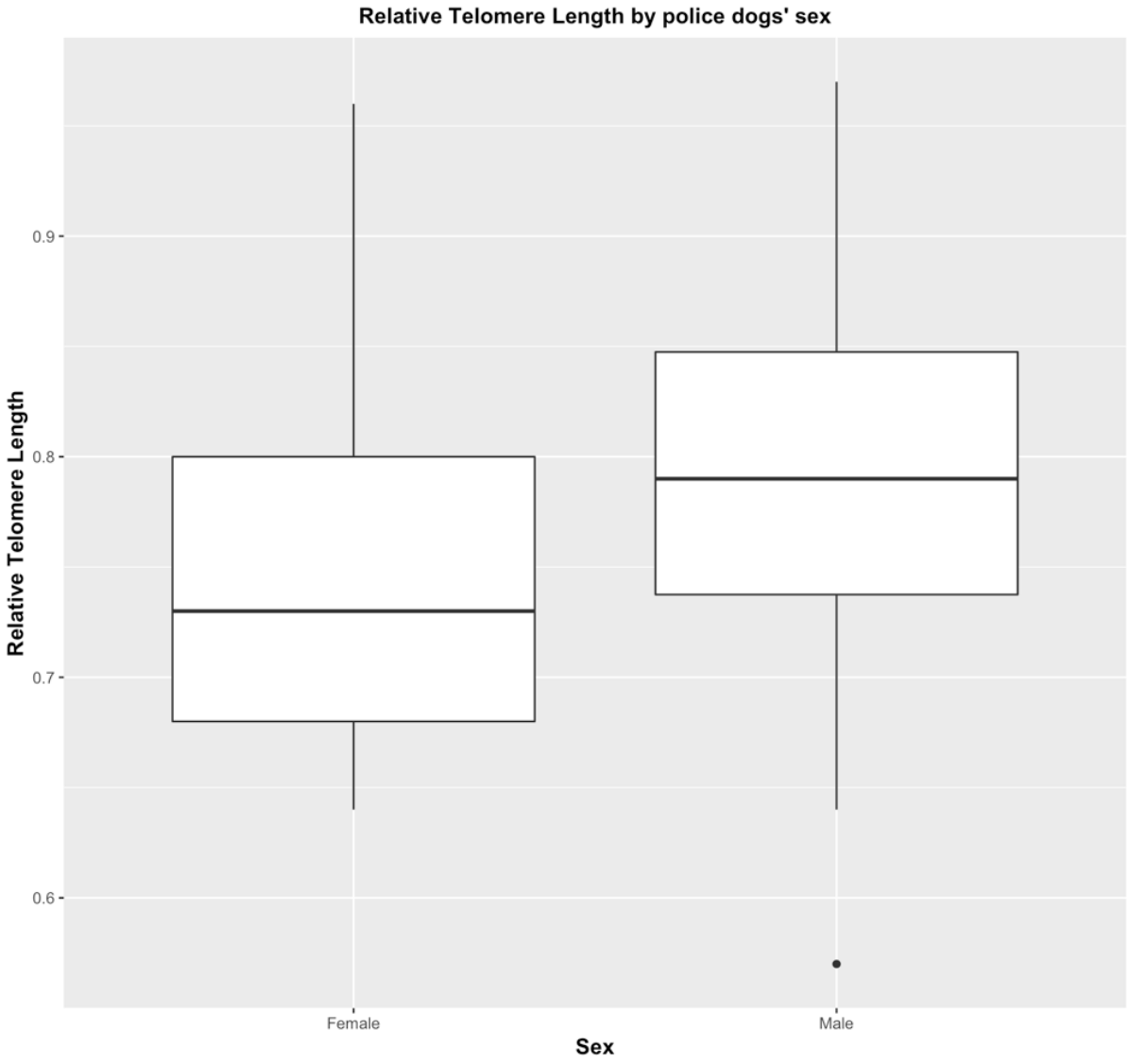

Figure 28 relative Telomere Length by police dogs' sex

4.6 Discussion

This study has shown that background/environment and its related husbandry topic dog management cause variation in relative Telomere Length. Considering that this is the first study exploring the effect of life background on relative Telomere Length the present results do indicate factors that should be further investigated when considering life experiences and biological ageing.

The discriminant analysis results were the first indications of how relevant the subject's background is when considering relative telomere length and cellular ageing. Although it is expected that telomere will be associated with age, the analysis showed that age was not an accurate predictor of relative Telomere Lengths, probably, because there was a significant difference between individuals' telomere lengths in the same age category. However, the discriminant analysis that used the dogs' groups to predict telomere sizes was highly accurate, which suggests that dogs that share similar routines and receive similar stimuli or social interactions have a similar cellular senesce. A previous study showed that dogs from different ages, breeds and sex from a shelter presented different learning rate and success for the same learning tasks, but all subjects consistently showed the same fear/submission behavioural trait. This lead the authors to emphasize the relevance of considering the differences of the dogs' backgrounds when training a dog (Weiss & Greenberg, 1997). Although a study investigating the behavioural status of military dogs detected that regardless of age, sex and breed: all individuals of the study displayed pacing as an abnormal behaviour and high cortisol levels, as signs of impaired welfare we found that police dogs had the longest telomeres (Lefebvre et al., 2009).

The previous analysis indicated that age was not accurate for predicting relative Telomere Length; to understand the factors that could impact telomere length the first model was run considering all background factors and all individuals had to be the same age. Once again results indicated how significant the subject's background was in influencing relative telomere length, this has already been showed in several studies; that is, environment plays a major role not only on physiological but also in dogs' behavioural responses (Bonne Beerda et al., 1998; Haverbeke, Diederich, Depiereux, & Giffroy, 2008; Titulaer, Blackwell, Mendl, & Casey, 2013).

The second model was run with the intention of verifying the correlation of a dog's body size and expected lifespan, that was already investigated by several studies (Fick et al., 2012; Mckevitt et al., 2018; Selman et al., 2013). However, no effect on relative telomere length was found when breed was considered or dogs' size, a result also found by Kraus and colaborators (2013) study, where the lack of effect of body size on senescence was explained with the hypothesis that deleterious effects of growth might be activated in earlier ages in large dogs. This study also highlights that the absence of the correlation between size and senescence might be simply due to sample size. It also stressed the need for a long-term, longitudinal study of senescence in a large cohort of companion dogs (Kraus et al., 2013).

The third model (in the Results) aimed to investigate all potential factors influencing relative Telomere Length. A dog's background category group was significant, showing that (Brazilian) laboratory dogs had lower relative telomere length when compared to the other groups. This result is coherent with studies that indicate that laboratory dogs because of their housing conditions, manipulation and social

isolation show higher rates of stereotypic behaviours and cortisol levels, which usually relate to low welfare conditions and poor quality of life (Bonne Beerda et al., 1999; Hetts, Clark, Calpin, & Arnold, 1992; Wojciechowska & Hewson, 2005). Males presented a longer relative Telomere Length when compared to females, conversely studies with rats and humans showed that females presented longer telomeres than males (Cherif, Tarry, Ozanne, & Hales, 2003; Mayer et al., 2006).

Dog sleeping site was associated with telomere length where dogs who slept in bed with their owners had longer telomeres than dogs that were inside the house but not allowed in the bed. Studies investigating dogs co-sleeping with owners are scarce, however, studies investigating the effects of owners co-sleeping with their dogs are proving positive effects from this association, where women who slept with their dogs had less disruption to their sleep and lower prevalence of bad dreams (Hoffman et al., 2018). The only study considering the effects of co-sleeping on dogs showed that the animals who slept with their owners were classified as more anxious than dogs who did not co-sleep but stated that more complete population data and data from across a dog's lifetime is needed to a better understanding of the effects of co-sleeping on dogs (Cannas et al., 2018).

Dogs that were walked every day presented the lowest telomere length even when compared with dogs who were never walked. Because we did not collect information regarding the duration of walks, the number of walks per day, if the dog was walked on leash or off leash we cannot state the cause of this negative correlation between activity and telomere length. However, a study about the use of antioxidants highlighted that the superoxide radical, a by-product of oxygen metabolism

and very intensive exercise, causes many types of cell damages, including an acceleration of age-related muscle mass loss and a reduced lifespan in humans (Simioni et al., 2018).

Dogs that have contact with 1-2 other animals showed greater telomere length when compared to dogs that have no contact to other animals. Mertens and Unshelm (1996) found that being isolated from other dogs prevented dogs from fulfilling their social interaction needs. This resulted in single housed shelter dogs showing behavioural stereotypies, while group-housed dogs did not express any stereotypical behaviour. A study with African grey parrots, found those housed in pairs had longer telomeres than single housed birds, showing that social isolation shortens African grey parrots' telomeres (Aydinonat et al., 2014). Dogs that were housed with more than 5 other animals showed shorter telomere lengths, this could be due to the potential increase of social conflict. A study showed that dogs sharing their environment with more dogs, that not were often exposed to social confrontations, not necessarily lead to fights, but to be resolved individuals needed to read each other's ritualized behaviour (Mertens & Unshelm, 1996). A plausible explanation would be that one or two dogs can have a social enrichment role, whereas more than five dogs could mean competing for resources and space in the house.

There is increasing interest in understanding how and why telomeres become shorter since this mechanism triggers cell senesce. Many factors behind this process still need to be investigated but several studies have already shown that the telomere length is associated with age, the older the subject are the shorter the telomere length (Ding, Mangino, Aviv, Spector, & Durbin, 2014). There are studies indicating

telomere shortening with older age in dogs and its association with a breed's predicted lifespan, the results from Model 2 did not find any effects of age or breeds on the telomere length; the reason for absence of these associations in the present study remains unclear but could be due to the heterogenous nature of our sample (Buddhachat et al., 2017; Fick et al., 2012; Mckevitt et al., 2018).

The fourth statistical model investigated the difference between police dogs from Brazil and from the UK; although there were several differences between the two groups the only factor that significantly correlated with telomere length was the different types of dog occupation. Dogs that work with search and rescue had the shortest relative Telomere Length in comparison to dogs who perform other work types. Police dogs are usually trained for security, patrol, or detection, but they can be trained for more than one task and work in a variety of situations. Search dogs can be trained to detect only one scent: from either of live or dead victims or to detect both live and dead victims scents, they call those cross-trained dogs (Lit & Crawford, 2006). There is logistic dilemma about employing cross-trained dogs in a catastrophe with both kind of victims (scents) or of having a dog that is trained for work in only one case scenario. A study reported that cross-trained search dogs have inferior performance in the task of signaling only live victims when compared to live-only trained dogs when cadaver scent was present in the scenario (Lit & Crawford, 2006). The authors suggest that the lower performance could be due to the potential confusion of the dog when asked to alert for live people and withhold learned operant behaviors - responding to cadaver scent (Lit, Schweitzer, & Oberbauer, 2011). In this case alerting the handler for the non-requested, but learnt and trained scent, illustrate a situation where the dog is not going to be rewarded,

the absence of reward induces the paradigm on inequity aversion, which in dogs results in them becoming stressed for not receiving what is 'fair' (Range, Horn, Viranyi, & Huber, 2009).

Considering this as the first study investigating the role of the background on dogs' telomere lengths, the results obtained, even though in many aspects did not presented statistical significance, were sufficient to indicate directions for future research. Although in total our sample size was significant, having dogs from different backgrounds, sex and ages diluted the general sample size resulting in few or no individuals for certain group, sex and age categories, which could have influenced the possibility of finding more trends in potential factors affecting relative telomere length (i.e. type II statistical errors – false negatives). Another aspect that difficult the comparison among pet dogs, for example, and was highlighted by Cafazzo and collaborators (2014) is that there is an extraordinarily high number of variables that characterize a dog's household environment. Future studies need to consider more homogenous sampling to detect factors influencing premature aging. For example, dogs produced by The Guide Dogs for the Blind all are bred and reared in the same housing conditions until they are 6 months old, a setting that would be ideal to investigate relative telomere length variations between sex and age for example, without considering nutrition, activity, sleeping site and training that would be the same for all individuals. The alternative is to use very large samples sizes as per epidemiological studies of human aging and health (Duffy & Serpell, 2012).

Studies using animals' relative Telomere Length usually choose one aspect to investigate its effect on rTL such as: social isolation, ageing, cancer formation or re-

productive success, but none of them used multi background factors to understand its role on the telomere attrition as the present study did (Adams, Morgan, & Watson, 2018; Andrews et al., 2018; Aydinonat et al., 2014; Plot et al., 2012; Seeker, Ilska, Psifidi, Wilbourn, Underwood, Fairlie, Holland, Froy, Salvo-Chirnside, et al., 2018).

Chapter 5 Evaluating laboratory dogs' relative Telomere Length over one year

5.1 Introduction

Due to the large number of animals used in laboratory research each year a simple and non-invasive method of assessing overall animal welfare status is required. Each year around 80 million animals are used in research in the world, in the UK 3.79 million procedures were carried out involving living animals, the majority of experiments were performed with rodents (70%), then with fish (16%), birds (7%) and dogs (1%). Dogs (*Canis familiaris*) were in the category of specially protected species and 3,847 individuals were involved in research in 2017 in the UK (Department of Science and Innovation, 2017). Dogs, cats, horses and primates are classified under the category 'Specially Protected Species' because as they are afforded additional protection according to The Animals (Scientific Procedures) Act 1986 (2012). As many people share a special bond with dogs and have them as companion animals some countries such as the UK give them special protection in laboratories, which means that researchers need to provide strong justification to use dogs and not substitute them for other species in their experiments (UK Home Office, 2018).

Debates on how to measure welfare of research animals concern how to minimise discomfort, benefit the animals and the experimental scientific outcomes. Assessment of laboratory animal welfare were initially developed based on the 3 Rs (i.e. replacement, reduction and refinement; Tannebaum, 2015) and then on the five freedoms from the Animal Welfare Act 1996, which states that an animal must be

free from hunger, from discomfort, from pain or injury, from fear and to express their normal behaviour (Brambell Committee, 1965). Because the term welfare relates directly to the state of an individual as it is associated to its environment, the laboratory setting and routine can be challenging and restrictive for animals (Broom, 1991; Fraser et al., 1997). The three main approaches used in laboratory settings are: a) monitoring individuals' behaviours, noting any change in normal behaviour and presence of sudden aggression, locomotor stereotypes, auto-mutilation, b) monitoring individuals' body conditions (i.e. weight loss, skin condition), c) enhancing housing conditions, considering sufficient space for nesting, resting, foraging and social contact (Baumans, 2005; Dean, 1999; Meagher, 2009).

Studies have elements that have negative impacts on laboratory dog welfare such as the lack of exercise, which is usually related to small or barren housing, and the lack of socialization (Coppinger & Zuccotti, 1999). Because of their social nature, social contact is extremely important for dogs' wellbeing and studies indicate that they value equally interactions that come from another dog or from a human (Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Phillips, et al., 2007).

Similarly, to wolves (*Canis lupus*), dogs are socially interactive animals, but because of the domestication process they had their human-communicative skills enhanced. Social interaction abilities are needed by dogs for developing many skills from problem solving to adequate behavioural responses towards another dog (Miklósi, 2015). For example, dogs raised in confinement may develop abnormal behaviours of extreme fear, atypical aggression, or develop stereotypies (Shepherdson, Mellen, & Hutchins, 1998). Hubrecht (1993) pointed out that small

increases in the laboratory dog's opportunities for social interactions with humans or with conspecifics is enough to reduce abnormal chewing on housing furniture.

Currently laboratory dogs are mainly housed in indoor individual pens for many logistical reasons such as kennel cleaning and managing the individual for experiments, observing their reactions and side effects after a procedure, but also to avoid any possible environment contamination, or lack of control of climate; for example, in the case they were housed outside (Coppinger & Zuccotti, 1999). Spangenberg and collaborators (2006) conducted research with laboratory dogs comparing the dog's behaviour in individual indoor housing and dogs in individual outdoor housing and the results indicated that dogs with outdoor access spent more time in voluntary activity and displaying investigative behaviours without altering their health status. Scientists agree that when outside housing is not possible it is important to enrich dogs' environment with objects that possess biological relevance for the species, in case of dogs, social housing can play a major role as a enrichment (Bayne & Würbel, 2014; Coppinger & Zuccotti, 1999; Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Phillips, et al., 2007; Wells, 2004).

Telomere attrition is biomarker of biological ageing because telomere length reduces with age. Stress plays a major role in the organism; high levels of constant stress is one of the factors that leads to premature ageing, whereas low levels of stress are associated with longevity and good welfare (Adams et al., 2018; Bateson, 2016; Plot et al., 2012). For this reason, telomeres have been proposed as a biomarker that integrates the impacts of different kinds of stress (good and bad; Kloet, 1999; Aschbacher, 2013) and adversity into a common currency being indicated as

good marker of either good welfare or bad welfare. This is because it can give deeper insights of the impact husbandry regimes have on animals' quality of life be they positive or negative (Pepper,2018; Wolfensohn, 2018).

Most studies regarding telomere length developed with non-human subjects are with animals in a laboratory setting. Studies investigating how stress, chronic inflammation, cancer and cell senescence are related to relative Telomere Length and telomere lengthening have been developed with mice as subjects (Espejel et al., 2004; Herrera et al., 1999; Kondratov, 2006; Passos et al., 2014; Xie et al., n.d.). Although the technique to measure telomeres is already being used in laboratory animals to investigate questions related to longevity, no study was found that used telomere length as a welfare parameter under commercial laboratory conditions. The present study addresses gaps in the literature by using relative Telomere Length as a tool to assess a laboratory dog's welfare over time.

5.2 Objectives

Investigate whether dogs' telomere length present in buccal cells was affected by social enrichment over a one-year period.

5.3 Justification

The change over time in rTL is already established for humans as a marker of wellbeing; however, the increase of relative Telomere Length is new and not completely understood for human subjects, and has not been explored in the animal welfare field yet (Bateson, 2016; Gotlib et al., 2015).

5.4 Methods

5.4.1 Laboratory Dogs

We sampled 13 entire adult dogs, 7 males (3.86±2.37 years old) and 6 females (3.65±0.96 years old) from a laboratory environment in 2017 and 2018 (*Table 21*). These dogs are from a laboratory facility belonging to a university in Brazil and used as a breeding stock, none of the dogs was involved in any research activity, they were all healthy and had the same feeding schedule. These dogs were all mixed breeds and were not walked (*Figure 29*).

Table 21 Information from 2017 data collection of dogs from a laboratory setting in Brazil

ID	Sex	Age (years)	Weight (Kg)
F01	Female	6	18.5
F02	Female	6	17.7
F09	Female	3	20.8
F10	Female	5	17.3
F13	Female	6	31.5
M01	Male	3	21.3
M02	Male	3	19.8
M04	Male	3	32.4
M05	Male	3	26.5
M06	Male	6	22.2
M07	Male	6	25.8
M08	Male	3	26.7
M09	Male	3	29.4

Figure 29 Examples of dogs from the Veterinary School of University of Ouro Preto, Brazil.

5.4.2 Dog housing and husbandry from 2017 and 2018

In 2017 dogs had their kennel cleaned twice a day at 09:00 and 15:00 and were fed 500g of standard dog food (Croc Dog – Socil) after every cleaning. They did not routinely have exercise or social activities. Kennel's sizes were approximately 5.80m x 1.60m x 1.65m.

From April 2017 until May 2018 a behavioural study took place in the laboratory changing the dog's routine in two aspects: while the kennels were being fully cleaned the dogs were put together to socialize and have a play time, these sessions happened two to three times a week for approximately 30 minutes. The second aspect that changed was that dogs started to use a collar, which they were not habituated to wearing, for this reason researchers did a desensitizing process with the col-

lars through positive reinforcement and treats. As the collars needed to be checked every day, the dogs would see researchers every day, would be petted for approximately 10 minutes and receive treats.

We classified both changes in their routine as social enrichment (inter and intra-specific social interactions).

5.4.3 Swab collection

To associate cortisol levels and telomere length the dogs were orally swab sampled to collect buccal cells (i.e. DNA) following the sampling protocol as described previously in Chapter 2.

5.4.4 DNA extraction and quantification

The DNA extraction from the swab samples was performed using Buccalyse DNA Release Kit DNA following the manufacturer's protocol. Concentration of DNA extracted from swab samples was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA).

5.4.5 qPCR assay

A pool from the 2017 samples (conc. 91.7 ng/µl) and one for the 2018 samples (conc. 94.2 ng/µl) was produced. Each qPCR batch (i.e. run) consisted of two components: i) a tenfold point serial dilution prepared from pool of DNA from the 2017 pool or from the 2018, which was used to construct the standard curve and formed the basis of each primer optimization and quality control; ii) no template controls (NTC), which allowed for detection of potential contamination and/or primer dimer formation. This was done for the telomere gene and the reference copy gene.

Each step of the dilution series and the NTC were run in triplicate in each batch (Olsen, Bérubé, Robbins, & Palsbøll, 2012).

The primers, qPCR reaction mix and conditions were described previously in Chapter 2.

5.4.6 Measuring relative telomere length (RTL)

The measurement of the relative telomere length was calculated by using an adaptation of the qPCR method described by Cawthon (2002), as described previously in Chapter 2.

5.4.7 Statistical Analysis

We tested the normality of T/S ratios from each sample using Kolmogorov-Smirnov tests, differences between the T/S ratio means from 2017 and 2018 samples with paired t-test. All statistical analyses were performed using Minitab® 18.1.

5.5 Results:

5.5.1 Descriptive statistics for years 17 and 18

RTL samples from 2017 had a mean 0.68 [range: 0.61 to 0.74] and samples from 2018 had a 0.74 mean [range: 0.64 to 0.85]. All samples were successful in providing adequate DNA quantities (*Figure 30*).

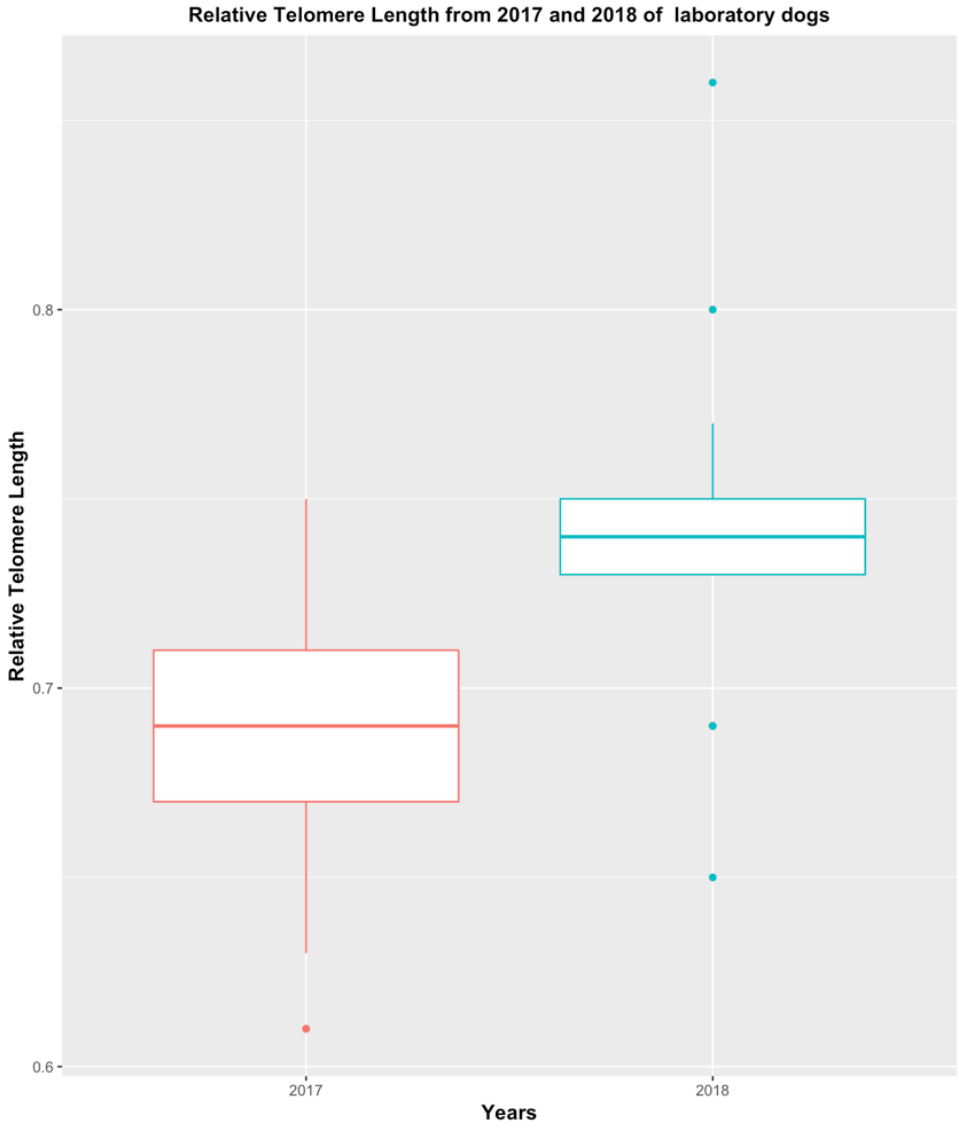

Figure 30 Median of relative Telomere Lengths from laboratory dogs from 2017 and 2018. Median: solid line; Interquartile range: boxes; Minimum and Maximum value: whiskers; Outliers: ●

5.5.2 Swab rTL were significantly different

A paired T-test indicated significant difference between 2017 and 2018 samples ($t(22) = -2.98$, $p < 0.001$). Female 9 (F9) and Female 10 (F10) had their relative Telomere Length decreased from 2017 to 2018. Female 13 (F13) and Male 20 (M20) did not had their relative Telomere Length changed over one year. All other nine

dogs had their relative Telomere Length increased over a one-year time period (*Figure 31*).

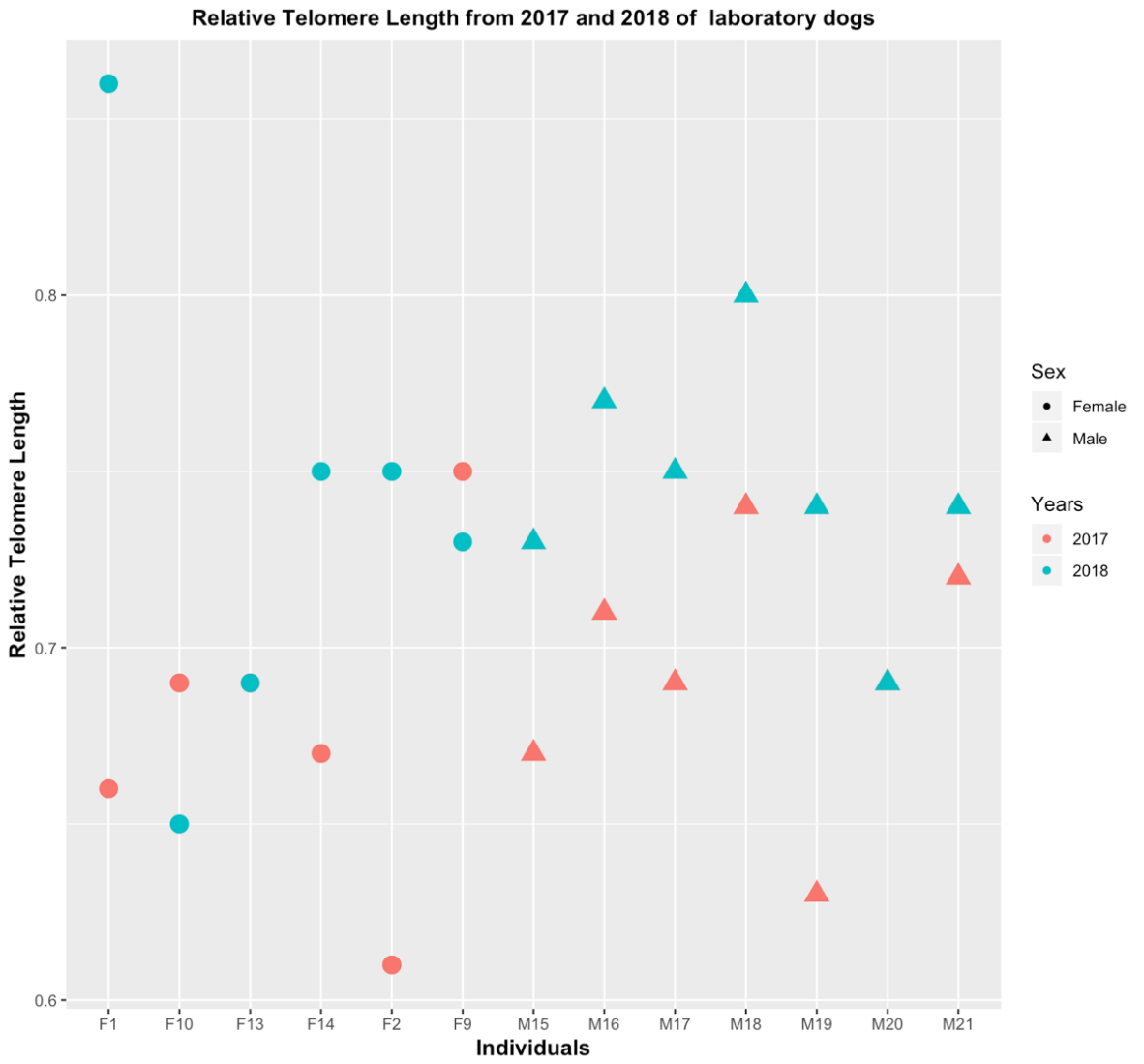

Figure 31 relative Telomere Length from female and male laboratory dogs sampled in 2017 and 2018.

5.6 Discussion

The current study indicated that relative Telomere Length in laboratory dogs can increase when dogs are exposed to social enrichment (Young, 2003). This promising result not only emphasises the use of relative Telomere Length as a practical method to access quality of life in dogs, but also highlights that positive experiences can impact on laboratory animals reducing premature aging due to stress.

The aspect that changed from 2017 and 2018 when the samples were collected was the establishment of playtime including all dogs of the section and short positive social interactions with researchers. Playtime involved interspecific socialization and exercise; some researchers agree that in the case of dogs socialization can be more important than physical exercise per se (Clark et al., 1997; Coppinger & Zuccotti, 1999).

The increase of dogs' relative Telomere Length under a period of socialization corroborates previous studies, which showed that laboratory dogs that had interspecific contact or were housed together showed less stereotypies, and less abnormal furniture chewing (Coppinger & Zuccotti, 1999). Remembering that naturally we would have expected telomere length to have reduced during the course of this study due to normal cell aging processes (Cherif et al., 2003). When interspecific socialization is not possible human contact, such as petting and play, could be considered as enrichment since studies have already shown that human contact has a positive impact on a dog's welfare (Gácsi, Dóka, Csányi, Topál, & Miklósi, 2005; Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Phillips, et al., 2007; Virányi et al., 2004).

Recent studies, in humans, showed that a healthy lifestyle, with exercise, healthy diet and mindfulness practices can mitigate the negative effects of stress on telomere length (Arsenis et al., 2015; Bateson, 2016; Boccardi et al., 2013). Several studies investigated the effect of meditation practice on telomere length, that is mediated by telomerase activity, and they found that not only the telomerase activity increased but was stabilized for four months and could stay stabilized for up to five years if meditators adhere to the practice (Conklin et al., 2018; Epel et al., 2016; Hoge et al., 2013). Although no study regarding telomere lengthening associated to positive practices have been undertaken using non-human subjects the aforementioned studies already indicate the potential of the approach when assessing an animal's life quality, especially for measuring positive animal welfare (Wolfensohn, 2018).

This is the first study to find telomere length growth/increase in a non-human species from an animal welfare perspective. Although our sample size was small, the results are sufficiently robust to indicate that minor social positive changes in a laboratory dog's routine (in terms of enrichment time) can have a major impact on their senescence. Future research in the animal welfare field should consider using telomere length as a tool for quality of life assessment for both positive and negative welfare.

Chapter 6 Evaluating Dogs' Welfare through 'apparent age'

6.1 Introduction

Ageing, like growth rate, may vary between individuals and for this reason, the concept of biological age as distinct from chronological age is accepted and extensively studied (Borkan & Norris, 1980). Biological age or 'apparent age' can be examined with photographs of the subjects by investigating features such as skin texture, skin pigmentation, eyes, mouth, hair colour (these are considered the most informative characteristics for humans; Gunn et al., 2008).

In humans, apparent age was used to predict mortality or age-related disease onsets, it is also associated with cortisol levels and telomere length (both indicative of stress levels) and used as a robust biomarker in clinical practice (Christensen et al., 2004; Noordam et al., 2012). Studies conducted with captive chimpanzees (*Pan troglodytes*) showed highly significant correlation between participant ratings for health and the healthiness of the individuals in a photograph (Kramer, 2012). Another study, with wild chimpanzees, showed that apparent age was related to bone loss in chimpanzees (Sumner, Morbeck, & Lobick, 1989). There are studies showing that some of the greatest pressures causing senescence in wildlife are predation and habitat loss (Monaghan et al., 2008; Williams, Day, Fletcher, & Rowe, 2006).

As in humans, maturing dogs experience typical physical changes such loss of muscle mass, graying and dull coat colour, pigment loss in nose and eye skin, development of cataracts; all these age changes progress alongside normal ageing

(Sanderson et al., 2014). A study conducted by King and colleagues (2015) indicates that physical change in a dog's face, hair greying, can be observed in young dogs that suffer from anxiety, thus, signalling premature ageing related to stress.

Studies have shown that having citizens analyse data increases the potential of expanding the scope and scale of research since non-expert volunteers are able to produce accurate and reliable data when classifying images (Swanson, Kosmala, Lintott, & Packer, 2016). Because dogs live in familiar proximity to humans, they represent great candidates for studies involving citizen science, they are present in 40% of United States households, and in 2011 it was estimated that were 9.4 million companion dogs in the UK making them perfect science subjects to expand the frontiers of public engagement (Asher et al., 2011; Wynne, 2009).

Accelerated telomere attrition is associated with pathologies such as cardiovascular disease, diabetes and with habits such as poor diet, lack of exercise and psychological stress (Abrahim et al., 2019; Choi et al., 2008; Mirabello et al., 2019). Chronic psychological stress leads to high levels of cortisol secretion (i.e. stress hormone), which is associated with a higher perceived age as cortisol have severe effects on tissues, particularly skin ageing, a crucial element of facial aging that is consequently associated with apparent age (Gunn et al., 2009; Noordam et al., 2012). Christensen and collaborators (2009) discussed how the comparison between the perceived age and chronological age can be used as indicator of health because biomarkers of ageing can suggest several common health risks besides relating to cellular ageing and telomere length.

Although the aforementioned studies have associated chronic stress and ageing, telomere length and ageing and even apparent age and telomere length, there are no studies with non-human mammals that associate all these three factors together. These parameters are known to be conserved in mammals, as Meyne and collaborators (1989) have shown that the (TAGGG) DNA sequence in the vertebrate species is derived from a common ancestor over 400 million years ago. Thus, we expect to find the same associations between dogs' relative Telomere Length and perceived age; thereby allowing us to try and validate apparent age as an animal welfare measuring tool (but obviously not a tool that measures instantaneous animal welfare level).

6.2 Objectives

Investigate how people with different canine expertise assess the apparent age of dogs.

Investigate if the apparent age of dogs is related with their relative telomere length.

6.3 Justification

Currently, animal welfare assessment uses behavioural and physiological parameters, which can be time-consuming and also expensive. Here the potential of a new tool for welfare assessment that could be used by non-specialists in different situations was investigated. The ability to determine animal welfare through a photograph could be useful in future for pet owners, veterinary surgeons, kennel owners and dog shelter managers.

6.4 Methods

6.4.1 Dog subjects

We took photographs from every dog sampled previously in Chapter 3, in total 262 domestic dogs were photographed. Among the domestic dogs we sampled were pet dogs, shelter dogs, police dogs, laboratory dogs, rehomed dogs and behavioural research dogs.

6.4.2 Photograph collection, treatment and classification

Several photographs were taken for each individual, using a Nikon D7200 Digital SLR; the two highest quality images of each individual looking at the camera were selected. These images were cropped to show only the head, with a small amount of neck/body and background landscape (Kramer, 2012). When necessary, the software Adobe Photoshop© CC 2015 and Adobe Lightroom© were used to blur backgrounds and enhance picture focus. The goal was to highlight the dog and prevent other elements of the photo from interfering with the assessment. Dog pictures were grouped by their age: 0-2 years old were put in the group Young; 2-5 years old were in Adults group; and older than 6 years old were labelled as Seniors. Dogs that were in between ages (i.e. 3 years and 3 months or 3 years and 7 months) had their age rounded, down if younger than six months and up if older that six months.

6.4.3 Evaluation of photographs by specialists

We invited researchers, veterinary surgeons and dog trainers to evaluate the photographs. Information regarding the assessors' sex, age, job and experience (time) with dogs were collected. Assessors received the photos through an online

questionnaire on the platform Google Forms with 60 dog pictures provided, where the assessors could rate anonymously and no information regarding the individual canids was given. Assessors were asked to consider age of the individuals in the photographs and classify the dogs as young, adult or senior. Each photograph was assessed by 53 assessors.

6.4.4 Evaluation of pictures by general public volunteers

To contrast the evaluation from the specialists with members of general public we use the online platform Zooniverse. The dog's photos were uploaded in the web-site and were shown randomly to volunteers where they could rate anonymously and no information regarding the individual canids was given. The Zooniverse platform collected only the classification volunteers made so no demographic data regarding volunteers age, sex or experience time with dogs were obtained. The same 60 dogs' photographs assessed by specialists were evaluated by 33 volunteers.

6.4.5 Building Zooniverse Project: Dogs' Life Project

The Zooniverse is a free platform for people-powered research, where re-search is made possible by volunteers, the research can be assed either by the plat-form volunteers or by selected volunteers that receive a link to access the project. The website enables researchers upload different media type (e.g. videos, camera trap photographs), create a dataset and build a classification system that will work for the research.

The platform offers a tool called Project Builder where the researcher can work on the three key elements of the project: the main page, where basic infor-

mation about the researchers and the aim of the project will be shown; the workflows, that are the sequences of the tasks that volunteers will be asked to do and the subject sets, that are the collection of images that volunteers are questioned to complete tasks on. In addition, the project builders can add a tutorial explaining how the volunteers are expected to perform the task and a help tab can also be added with extra information for each task.

The Dogs' Life Project was built in August of 2018 and, after pilot testing, volunteers started to evaluate the pictures in November of 2018. All 262 pictures were uploaded, and each of them were set to be evaluate by up to 50 different people. Volunteers needed to classify dogs as 0-2 years old, 2-5 years old or older than 6 years old, respectively equivalent to young, adult and senior categories from the specialist's analysis. All elements of the Dog's Life Project build (i.e. project main page, team information, tutorial and tasks' help) are in Appendix 7.

6.4.6 Dogs' relative telomere length (RTL)

For comparing the relative Telomere Length with the pictures, we used the dogs that were common assessed from both groups of assessors and the relative Telomere Length that were calculated in Chapter 4. For the expected relative Telomere Length by age and confidence interval; was used the telomere mean values for each age, that were calculated with descriptive statistics in Chapter 4. Not all dogs that had their age assessed by photographs had their telomere measured in the previous Chapter, therefore these dogs were not accounted for the comparison between apparent age and relative Telomere Length.

6.4.7 Data Analysis

The first overall analysis for the 60 dogs was a percentage score calculate from all correct predictions from each dog, from this percentage it was calculated the probability of a dog be correct assessed. A discriminant analysis was performed to confirm if the dogs grouping by their age category could be predicted by in the assessors correct apparent age evaluations.

Then from the 60 dogs evaluated we selected the top 10 dogs correctly assessed and top 10 dogs most wrongly assessed by specialists and by Zooniverse volunteers to evaluated dogs' age category.

A Generalised Linear Model was used to investigate if dogs' age, sex and origin category (i.e. shelter, pet, work) had any influence on the correct age predictions from specialists and Zooniverse volunteers.

Another Generalised Linear Model was used to identify if the specialist's sex, age, job and experience time with dogs had any influence on their success predicting dogs' ages. To obtain the optimal model the function drop1 was used to remove all non-significant variables, all models were then checked using the Akaike's information criterion (AIC) and then the most explanatory model was chosen.

Responses from specialists and from Zooniverse volunteers were firstly analysed separately, then the answers of all participants considered together for the question regarding factors that helped or difficulted the assessor's dog age predictions.

All data were tested for normality (Kolmogorov–Smirnov Test) and the results of all statistical tests were considered significant at $p < 0.05$. Sixty dogs, twenty

young, twenty adults and twenty seniors were selected for this analysis. It is worth remembering that if guessing randomly we would expect the experts and volunteers to be correct 33.3% of the time as they were choosing from one of three categories.

All statistical analysis were run under the platform Studio R (RStudio Team, 2016) and Minitab 18 (Minitab Inc., 2010).

6.5 Results

6.5.1 Evaluating dogs' apparent age

Combining the assessments from specialists and Zooniverse volunteers' an average score for correct predictions for each dog was made. Probability showed that dogs that were assessed 28% correct and less are statistically significant (value less than expected), and 38% and more are statistically significant (values greater than expected) (*Table 22*).

Table 22 Mean percentage score for correct predictions of dogs' apparent age assessed from photos by specialists and volunteers

ID	Age	Sex	Category	Correct predictions (%)
SS45_M_2	Young	Male	Shelter	1.16
FPS60_F1.5	Young	Female	Pet	3.49
FPS117_F_10	Senior	Female	Pet	8.14
MPS12_M_12	Senior	Male	Work	10.47
FPS31_M_1	Young	Male	Pet	13.95
PS1_ M_7	Senior	Male	Pet	13.95
PS16_M_8	Senior	Male	Pet	16.28
WKDS12_F_5	Senior	Female	Rehome	18.60
FPS89_M_1	Young	Male	Pet	23.26
FPS50_F_3	Adult	Female	Pet	24.42
PBS10_M_2	Young	Male	Work	25.58
FPS113_M_7	Senior	Male	Pet	27.91
FPS87_M_4	Adult	Male	Pet	30.23
PBS34_M_6	Senior	Male	Work	30.23
PBS24_M_1	Young	Male	Work	31.40
FPS8_F_1	Young	Female	Pet	32.56
WS12_F_1	Young	Female	Work	32.56
WKDS18_M_1	Young	Male	Rehome	33.72
FPS26_F_2	Young	Female	Pet	34.88
WS14_M_1	Young	Male	Work	37.21
SS47_F_5	Adult	Female	Shelter	39.53
FPS29_M3	Adult	Male	Pet	40.70
FPS37_F_4	Adult	Female	Pet	41.86
FPS59_F_5	Adult	Female	Pet	41.86
FPS115_M_1	Young	Male	Pet	43.02
FPS36_F_8	Senior	Female	Pet	43.02
FPS56_M_10	Senior	Male	Pet	43.02
FPS90_M_2	Young	Male	Pet	43.02
PBS26_M_4	Adult	Male	Work	44.19
SS25_F_5	Adult	Female	Shelter	44.19

ID	Age	Sex	Category	Correct predictions (%)
MPS1_M_1	Young	Male	Work	45.35
MPS14_M_7	Senior	Male	Work	45.35
PS7_F_5	Adult	Female	Pet	45.35
WKDS19_F_2	Adult	Female	Rehome	45.35
PS8_F_5	Adult	Female	Pet	46.51
WS13_M_3	Adult	Male	Work	46.51
FPS57_M_4	Adult	Male	Pet	48.84
WKDS9_F_2	Young	Female	Rehome	50.00
FPS106_F_02	Young	Female	Pet	51.16
MPS19_F_3	Adult	Male	Work	51.16
FPS68_F_3	Adult	Female	Pet	52.33
WKDS24_F_5	Senior	Female	Rehome	52.33
FPS27_M_1	Young	Male	Pet	53.49
MPS15_M_14	Senior	Male	Work	53.49
MPS17_M_3	Adult	Male	Work	53.49
PBS16_M_5	Adult	Male	Work	54.65
FPS16_M_3	Adult	Male	Pet	55.81
PS13_M_2	Young	Male	Pet	55.81
FPS101_F_7	Senior	Female	Pet	56.98
SS5_M_2	Young	Male	Shelter	59.30
FPS4_F_9	Senior	Female	Pet	62.79
FPS48_M_4	Adult	Male	Pet	62.79
FPS28_F_3	Adult	Female	Pet	66.28
PS18_F_13	Senior	Female	Pet	74.42
FPS15_F_2	Young	Female	Pet	75.58
FPS73_M_7	Senior	Male	Pet	80.23
MPS11_M_ 12	Senior	Male	Work	81.40
FPS39_M_10	Senior	Male	Pet	90.70
PBS5_F_6	Senior	Female	Work	95.35
SS17_M_10	Senior	Male	Shelter	97.67

6.5.2 Dogs' apparent age evaluated by specialists

Sixty dogs were evaluated for apparent age prediction as young, adult or senior by 53 specialists, 43 women and 10 men and .70% of the respondents were between 25 and 39 years old. Biologists (3.77%), researchers (15.09%), veterinaries (7.55%), dog trainers (71.70%) and a student (1.89%) answered the questionnaire. When asked about how long they had been working with dogs 45.23% have been working with dogs for less than 5 years, 37.74% between 5 and 10 years and 5.665 have been working with dogs for more that 15 years. The correct guesses from specialists are summarised in (Table 23). To evaluate if the specialist's answers about the dog's age prediction grouped based on the dogs' age were accurate a discriminant analysis was performed using assessors correct answers as a predictor for grouping the dogs. The outcomes showed that the accuracy of the dogs' corrected assessed was 56.7% (Table 24).

Table 23 Specialists' frequency of correct answers for dogs apparent age prediction by dogs' true age category (N= 20)

Variable	Mean	SE Mean	StDev	Minimum	Median	Maximum	IQR
Young	20.65	2.48	11.11	0.00	20.50	42.00	17.50
Adult	25.50	1.43	6.39	14.00	25.00	39.00	6.50
Senior	28.25	3.59	16.06	3.00	28.00	52.00	31.00

IQR = interquartile range

Table 24 Discriminant analysis for specialists' correct answers for dogs apparent age prediction by dogs' true age category

	True Group		
Put into Group	Adult	Senior	Young
Adult	10	6	6
Senior	7	12	2
Young	3	2	12
Total N	20	20	20
N correct	10	12	12
Proportion	0.500	0.600	0.600

We selected the top 10 dogs' age correctly predicted by specialists, which included seven senior dogs, two adults and one young dog. Correct predictions from specialists are summarised in Table 25, where we can see that even the tenth dog with its age most accurate estimated was twice as likely to be correct in comparison with random chance.

Table 25 Dogs that had their ages most accurately estimated (%) from photographs by dog specialists

Dog ID	Age	Sex	Category	Correct predictions (%)
FPS101_F_7	Senior	Female	Pet	69.81
FPS28_F_3	Adult	Female	Pet	71.70
FPS48_M_4	Adult	Male	Pet	73.58
FPS15_F_1.33	Young	Female	Pet	79.25
FPS73_M_7	Senior	Male	Pet	81.13
MPS11_M_ 12	Senior	Male	Work	81.13
PS18_F_13	Senior	Female	Pet	81.13
FPS39_M_10	Senior	Male	Pet	96.23
PBS5_F_6	Senior	Female	Work	98.11
SS17_M_10	Senior	Male	Shelter	98.11

The top 10 dogs' age wrongly guessed by specialists included five senior and five young dogs. Wrong guesses from specialists are summarised in Table 26, where we can see that the tenth dog's least accurate assessment of age had his age 21% correct predicted compared to 33% for random chance.

Table 26 Dogs that had their ages least accurately estimated (%) from photographs by dog specialists

Dog ID	Age	Sex	Category	Correct predictions
SS45_M_2	Young	Male	Shelter	0.00
FPS60_F1.5	Young	Female	Pet	3.77
FPS117_F_9.5	Senior	Female	Pet	5.66
MPS12_M_12	Senior	Male	Work	9.43
FPS89_M_1	Young	Male	Pet	13.21
PS1_ M_7	Senior	Male	Pet	16.98
PS16_M_8	Senior	Male	Pet	16.98
WKDS12_F_5	Senior	Female	Rehome	18.87
FPS31_M_0.41	Young	Male	Pet	20.75
PBS10_M_2	Young	Male	Work	20.75

The specialists' predictions were normally distributed, so the first GLM model was performed with Gaussian distribution including dogs' age, sex and origin category (i.e. shelter, pet, work) as factors; no factor was found to be significant ($p > 0.05$).

A second model investigated if the specialists' characteristics had any influence on their success predicting dogs' ages. The specialists' predictions were normally distributed, so the GLM model was performed with Gaussian distribution including specialists' sex, age, job and experience time with dogs as factors. The optimal model achieved only the factor experience time with dogs as factors had a significant impact on the dogs 'age prediction, where professionals with more than 15 years of experience made more apparent age correct predictions ($R^2 = 0.190$, $p < 0.05$). Data are summarised in Table 27 and Figure 32.

Table 27 Final generalised linear model result for the effects of specialist's experience (time) with dogs on dog's apparent age prediction by dog specialists.

Parameters	Estimate±SD	t value	P
Intercept	45.557 ± 2.647	17.20	<0.0001***
Between 5 and 10 years [ab]	-1.223±3.018	-0.405	0.6872
For more than 15 years	11.667 ±4.585	2.544	0.0142*
Less than 5 years	2.291±2.960	0.744	0.4426

[1]Non-significant effects of parameters and interactions not shown were removed during the model selection process using the function drop1.

Best models for: Dog's apparent age prediction by dog AICc= 355.68

[a] Explanatory variables included in the full model: specialists' sex, age, job and experience time with dogs had sex

[b] Less than 5 years, Between 5 and 10 years; Between 10 and 15 years; For more than 15 years: Between 10 and 15 years

* P ≤ 0.01

*** P ≤ 0.0001

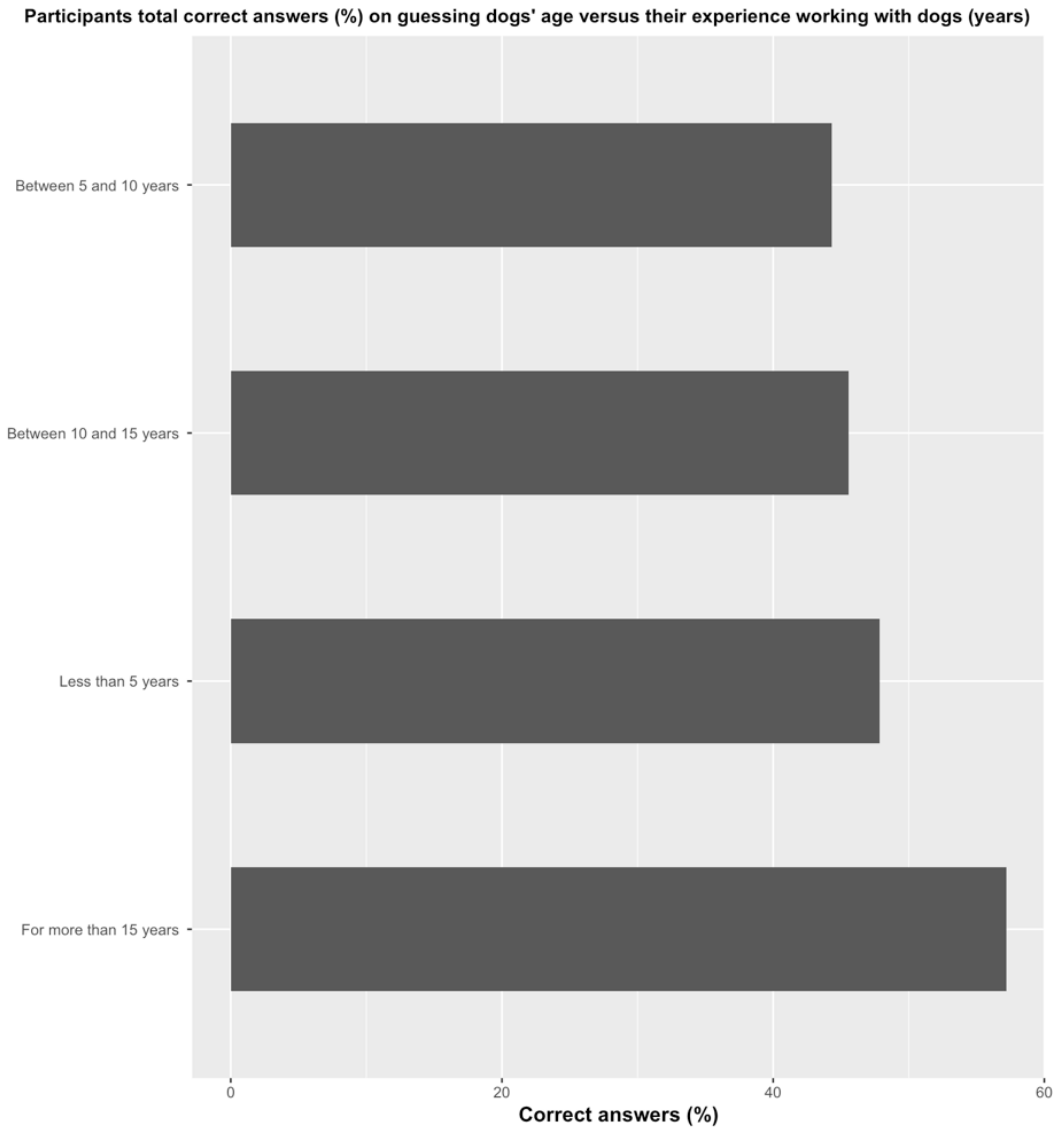

Figure 32 Dog specialists correct dogs age predictions (%) based on specialist's experience (time) with dogs.

6.5.3 Dogs' apparent age evaluated by Zooniverse volunteers

Sixty dogs were evaluated for apparent age prediction as young, adult or senior dogs by 33 volunteers, correct guesses from volunteers are summarised in (*Table 28*). To evaluate if the specialists' answers about the dog's age prediction were accurate a discriminant analysis was performed using assessors correct answers as a

predictor for grouping the dogs. The outcomes showed that the accuracy of the dogs' corrected assessed was 51.7% (*Table 29*).

Table 28 Volunteers' correct frequency of answers for each dogs' age category (N = 20)

Variable	Mean	SE Mean	StDev	Minimum	Median	Maximum	IQR
Young	11.45	1.52	6.80	0.00	12.50	23.00	9.50
Adult	14.75	0.909	4.06	7.00	15.00	23.00	5.75
Senior	14.85	2.15	9.60	3.00	12.50	32.00	19.00

Table 29 Discriminant analysis for volunteers' correct answers for dogs' age prediction by dogs' age categories

	True Group		
Put into Group	Adult	Senior	Young
Adult	5	3	4
Senior	9	13	3
Young	6	4	13
Total N	20	20	20
N correct	5	13	13
Proportion	0.250	0.650	0.650

We selected the top 10 correctly predicted by volunteers, which included seven senior, one adult and two young dogs. Correct predictions from volunteers are summarised in Table 30, where like with specialists' volunteers were twice as likely than by chance estimate correctly the age of the 10th most accurately aged dog.

Table 30 Dogs that had their ages most accurately estimated from photographs by Zooniverse volunteers

Dog ID	Age	Sex	Category	Correct predictions (%)
PS18_F_13.jpg	Senior	Female	Pet	63.64
FPS4_F_9.jpg	Senior	Female	Pet	66.67
WKDS9_F_2.jpg	Young	Female	Rehome	66.67
FPS68_F_3.jpg	Adult	Female	Pet	69.70
FPS15_F_1.33.jpg	Young	Female	Pet	69.70
FPS73_M_7.jpg	Senior	Male	Pet	78.79
FPS39_M_10.jpg	Senior	Male	Pet	81.82
MPS11_M_ .jpg	Senior	Male	Work	81.82
PBS5_F_6.jpg	Senior	Female	Work	90.91
SS17_M_10.jpg	Senior	Male	Shelter	96.97

The top 10 dogs' apparent age wrongly guessed by volunteers included five senior and five young dogs. Wrong guesses from volunteers are summarised in Table 31, which shows a very similar pattern to the specialists.

Table 31 Dogs that had their ages least accurately estimated from photographs by volunteers

Dog ID	Age	Sex	Category	Correct predictions (%)
FPS26-F_1.5.jpg	Young	Female	Pet	0.00
FPS31_M_0.41.jpg	Young	Male	Pet	3.03
FPS60_F_1.5.jpg	Young	Female	Pet	3.03
SS45_M_2.jpg	Young	Male	Shelter	3.03
PS1_ M_7 .jpg	Senior	Male	Pet	9.09
FPS117_F_9.5.jpg	Senior	Female	Pet	12.12
MPS12_M.jpg	Senior	Male	Work	12.12
PS16_M_8.jpg	Senior	Male	Pet	15.15
FPS113_M_7.jpg	Senior	Male	Pet	18.18
WKDS12_F_5.jpg	Senior	Female	Rehome	18.18

The volunteers' correct predictions were normally distributed, so a third GLM model was performed with Gaussian distribution including dogs' age, sex and origin

category (i.e. shelter, pet, work) as factors; none of the evaluated factors had effects on the volunteers correct predictions ($p > 0.05$).

6.5.4 Specialists versus Volunteers

A T-test was performed to evaluate difference between specialists (Mean= 46.8, Standard Deviation =22.8) and volunteers (Mean= 41.5, Standard Deviation= 21.9) success on predicting dogs age from photographs, but there was no significant difference between their scores ($t(117) = 1.30$, $p > 0.05$). From their top 10 correctly assessed dogs, seven dogs were common to both groups; these dogs were above the probability of being correctly assessed. From their top 10 wrongly assessed dogs, eight dogs were common to both groups, these dogs were below the probability of being correctly assessed.

6.5.5 What helped or difficulted assessors correctly predicting a dog's age

All 86 participants were asked about what features helped their predictions on dogs' age. The majority of participants considered important: dogs' hair colour (87%) and dogs' expression (61%). Only 6% of participants did not consider the described features as relevant to their predictions (*Figure 33*).

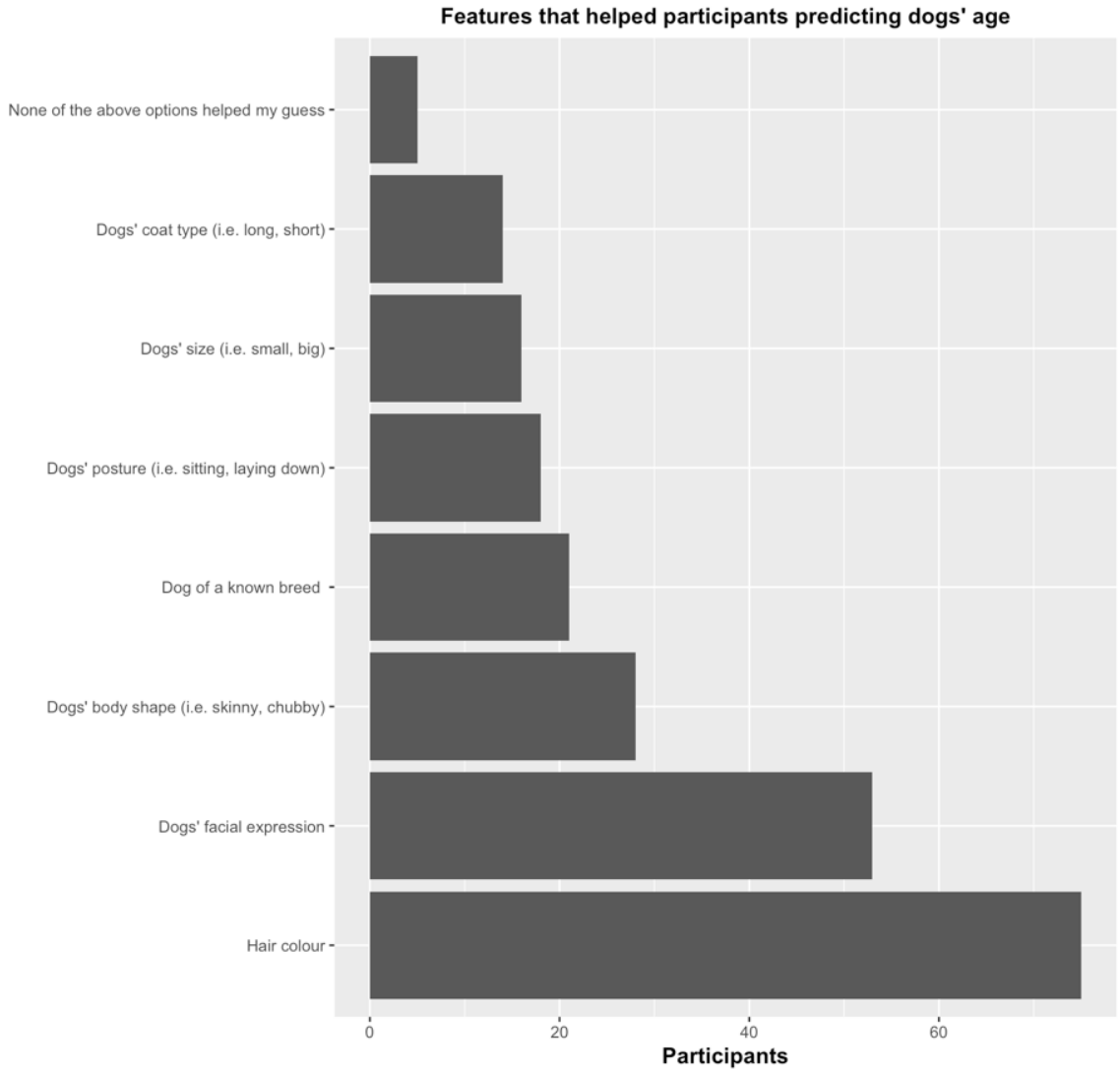

Figure 33 Features helped participants make predictions on a dog's age from photographs

Specialists were asked if any of the described features difficulted their predictions for dog's age from the photographs. Hair was the most problematic feature, 32% of the participants declared that hair type (i.e. short or long) made their prediction harder and 24% of participants said that hair colour also plays a negative role on the accuracy of their prediction. Less than a third of the participants considered that the photo background had a negative effect on their prediction (18%) *(Figure 34*).

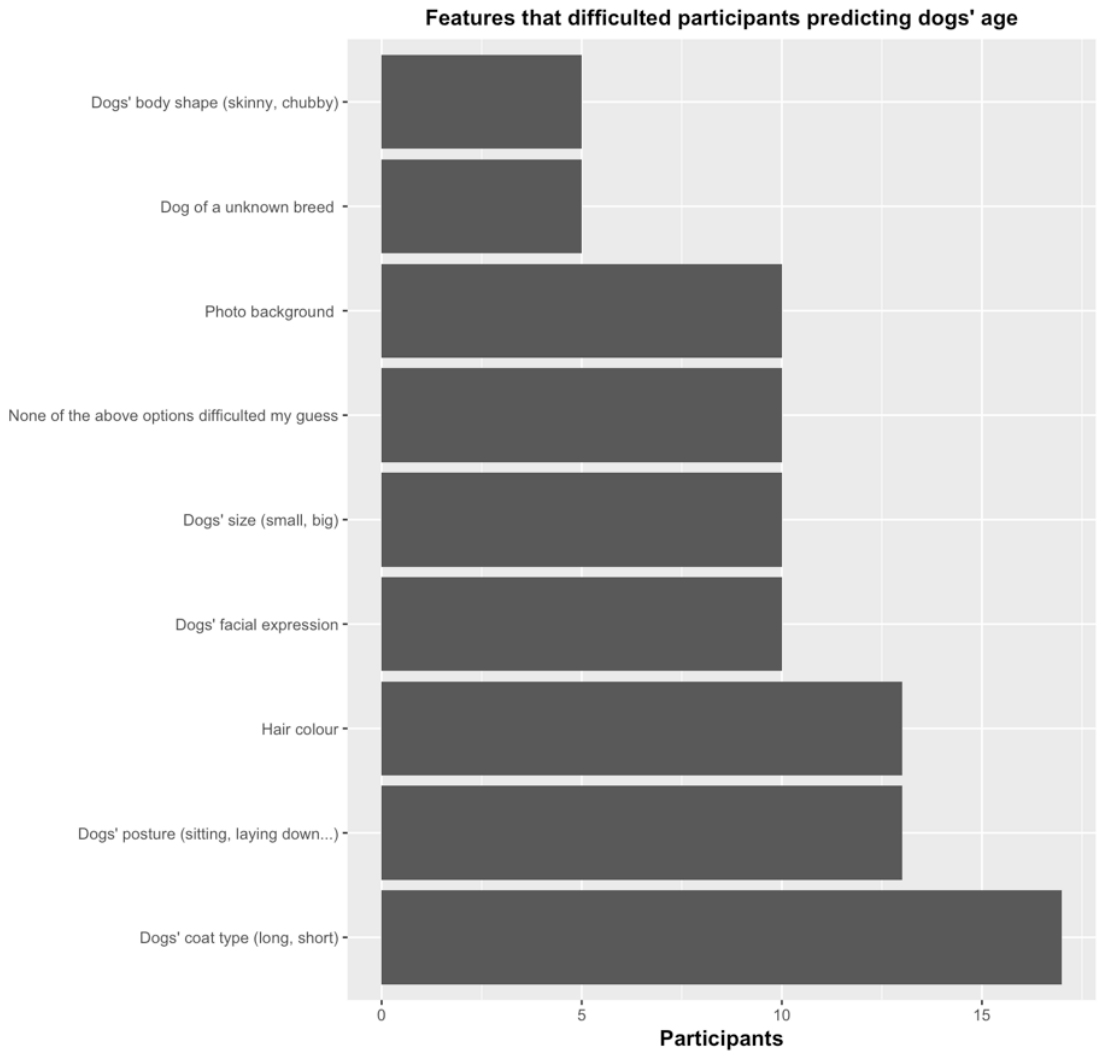

Figure 34 Features that difficulted participants' predictions on a dog's age from photographs

6.5.6 Dogs' apparent age versus Dogs' relative Telomere Length

We combined the data from the dogs that had their ages least and most accurately estimated by specialist and volunteers, with the relative Telomere Lengths that were measured previously in Chapter 3 (*Table 32*). Results from all dogs assessed in the current Chapter are in Appendix 11.

Table 32 Dogs' age prediction accuracy

Dog Id	Age	Mean		SE Mean	
		S	**v**	**s**	**v**
1	10	0.0566	0.1212	0.0320	0.0577
2	12	0.0943	0.1212	0.0405	0.0577
3	7	0.1698	0.0909	0.0521	0.0508
4	8	0.1698	0.1515	0.0521	0.0634
5	6	0.1887	0.1818	0.0543	0.0682
6	7	*	0.1818	*	0.0682
7	2	0.2075	0.3333	0.0562	0.0833
8	1	0.1321	*	0.047	*
9	2	0	0.0303	0	0.0303
10	7	0.8113	0.7879	0.0543	0.0723
11	12	0.8113	0.8182	0.0543	0.0682
12	6	0.9811	0.9091	0.0189	0.0508
13	13	0.8113	0.6364	0.0543	0.085
14	10	0.9811	0.9697	0.0189	0.0303
15	9	*	0.6667	*	0.0833
16	7	0.6981	*	0.0637	*
17	2	0.7925	0.697	0.0562	0.0812
18	2	*	0.6667	*	0.0833

S = Specialist group N = 53. Dogs 6, 15,18 were not evaluated by this group
V = Volunteer group N = 33. Dogs 8 and 16 were not evaluated by this group.

The group of the top nine dogs with their age most accurately predicted had seven senior dogs and two young dogs (*Table 33*) (*Figure 35 – 45*). Five of the seven senior dogs had shorter or the expected relative Telomere Length (rTL) for their age, two senior dogs had longer relative Telomere Length than the expected for their age. One of the young dogs had the expected relative Telomere Length for her age and the other had longer relative Telomere Length than the expected for their age. It is worth noting that all telomere lengths are within the predicted confidence interval limits.

Table 33 Dogs that had their age most accurately predicted from photographs by specialists and volunteers

Dog Id	Age	Sex	Category	Indiv. rTL	rTL Expected for Age	CI (95%)
1	Senior	Male	Pet	0.747	0.742	(0.658, 0.829)
2	Senior	Male	Work	0.570	0.674	(0.442, 0.904)
3	Senior	Female	Work	0.820	0.751	(0.710, 0.774)
4	Senior	Female	Pet	0.646	0.792	(0.660, 0.939)
5	Senior	Male	Shelter	0.792	0.745	(0.714, 0.787)
6	Senior	Female	Pet	0.724	0.752	(0.710, 0.774)
7	Senior	Female	Pet	0.736	0.742	(0.729, 0.770)
8	Young	Female	Pet	0.744	0.750	(0.722, 0.771)
9	Young	Female	Rehome	0.846	0.750	(0.729, 0.770)

CI = Confidence Interval (95%) – descriptive statistics calculated for each age from chapter 4.

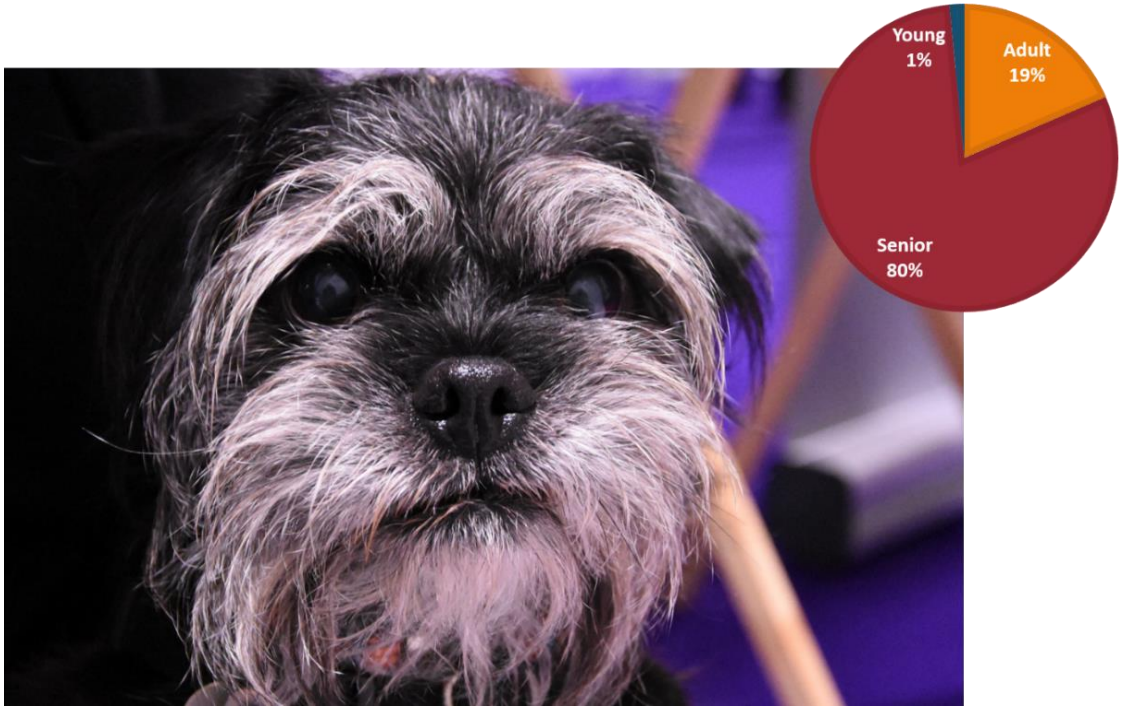

Figure 35 Dog 1: Senior male, seven years old mainly classified as a senior dog by assessors. Telomere length was as expected for his age (Dog 1 relative Telomere Length: 0.75; Expected relative Telomere Length for the age: 0.74).

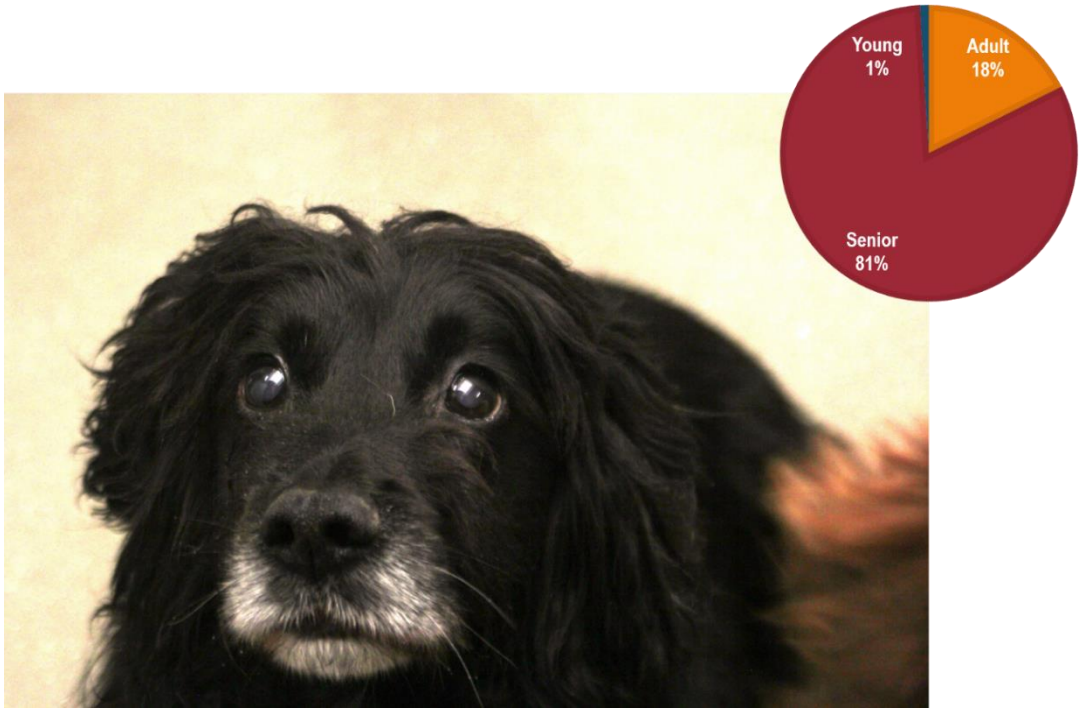

Figure 36 Dog 2: Senior male, twelve years old mainly classified as a senior dog by assessors. Telomere length shorter than expected for his age (Dog 2 relative Telomere Length: 0.57; Expected relative Telomere Length for the age: 0.67).

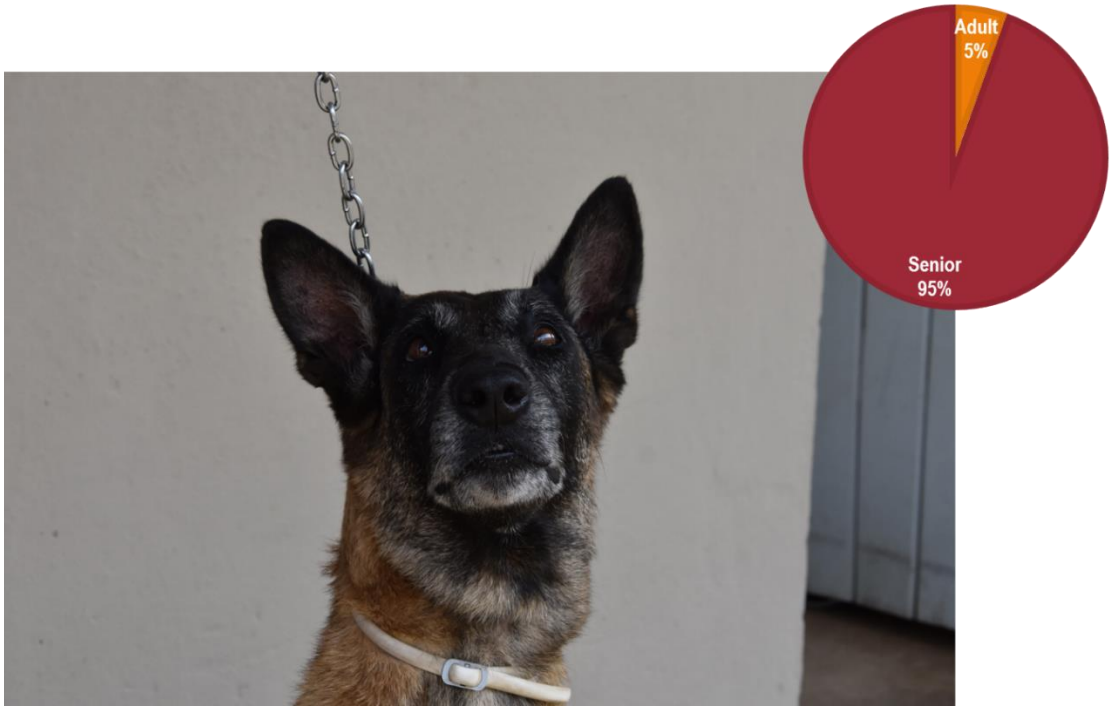

Figure 37 Dog 3: Senior female, six years old mainly classified as a senior dog by assessors. Telomere length longer than expected for her age (Dog 3 relative Telomere Length: 0.82; Expected relative Telomere Length for the age: 0.75).

Figure 38 Dog 4: Senior female, thirteen years old mainly classified as a senior dog by assessors. Telomere length shorter than expected for her age (Dog 4 relative Telomere Length: 0.65; Expected relative Telomere Length for the age: 0.79).

Figure 39 Dog 5: Senior male, ten years old mainly classified as a senior dog by assessors. Telomere length longer than expected for his age (Dog 5 relative Telomere Length: 0.65; Expected relative Telomere Length for the age: 0.79).

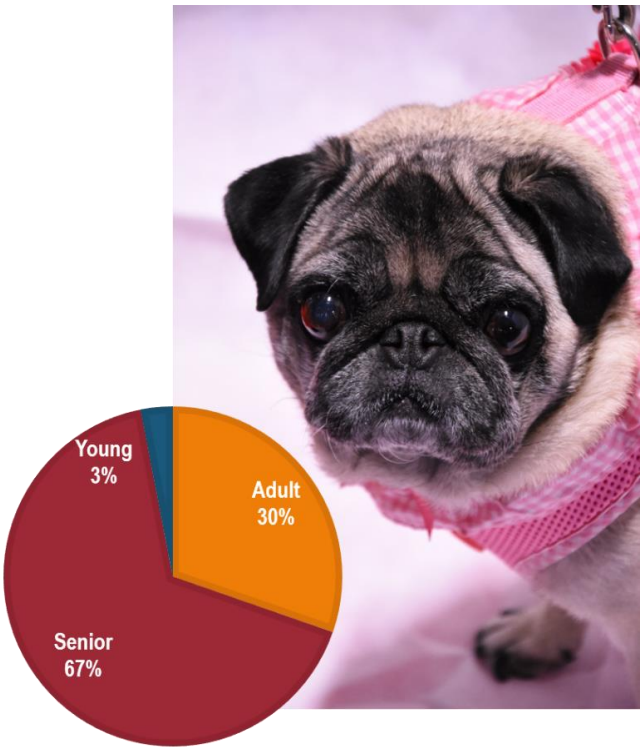

Figure 40 Dog 6: Senior female, nine years old mainly classified as a senior dog by assessors. Telomere length as expected for her age (Dog 6 relative Telomere Length: 0.72; Expected relative Telomere Length for the age: 0.75).

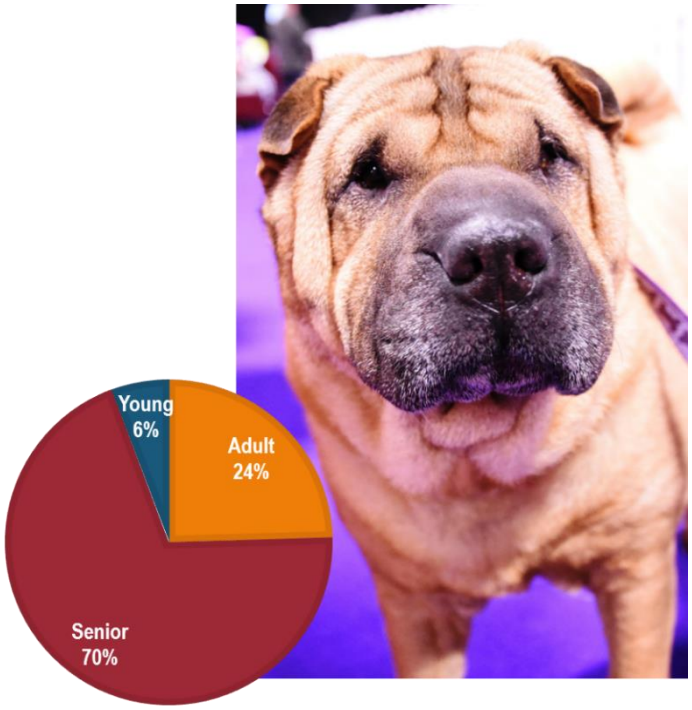

Figure 41 Dog 7: Senior female, seven years old mainly classified as a senior dog by assessors. Telomere length as expected for her age (Dog 7 relative Telomere Length: 0.74; Expected relative Telomere Length for the age: 0.74).

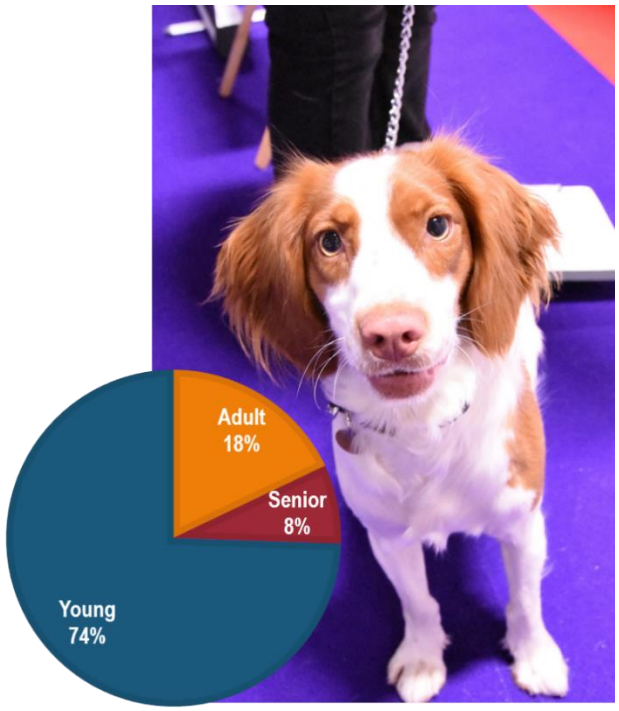

Figure 42 Dog 8: Young female, two years old mainly classified as a young dog by assessors. Telomere length as expected for her age (Dog 8 relative Telomere Length: 0.74; Expected relative Telomere Length for the age: 0.74).

Figure 43 Dog 9: Young female, two years old mainly classified as a young dog by assessors. Telomere length longer than expected for her age (Dog 9 relative Telomere Length: 0.85; Expected relative Telomere Length for the age: 0.74).

The group of the top nine dogs with their age least accurately predicted had six senior dogs and three young dogs (*Table 34*) (*Figure 44– 53*). Four of the six senior dogs that were classified as young or adult by the assessors had longer telomeres than the expected relative Telomere Length for their chronological age, two senior dogs assessed as young or adults had shorter relative Telomere Length than the expected for their chronological age. All three young dogs that were classified as adult or senior by assessors had shorter relative Telomere Length than the expected relative Telomere Length for their chronological age. It is worth noting that the relative Telomere Length of four of these dogs was outside of the confidence interval limits and two more were on the limits of the predictions.

Table 34 Dogs that had their age least accurately predicted from photographs by specialists and volunteers

Dog ID	Age	Sex	Category	Predicted	Indiv. rTL	rTL Expected for Age	CI (95%)
10	Senior	Female	Pet	Adult	0.683	0.745	(0.710, 0.774)
11	Senior	Male	Work	Young	0.751	0.674	(0.442, 0.904)
12	Senior	Male	Pet	Adult	0.776	0.742	(0.714, 0.787)
13	Senior	Male	Pet	Adult	0.902	0.801	(-1.047, 2.637)
14	Senior	Female	Rehome	Adult	0.811	0.751	(0.658, 0.829)
15	Senior	Male	Pet	Young	0.670	0.742	(0.695, 0.804)
16	Young	Male	Work	Adult	0.706	0.750	(0.695, 0.804)
17	Young	Male	Pet	Adult	0.640	0.746	(0.729, 0.770)
18	Young	Male	Shelter	Senior	0.712	0.750	(0.729, 0.770)

CI = Confidence Interval (95%) – descriptive statistics calculated for each age from chapter 4.

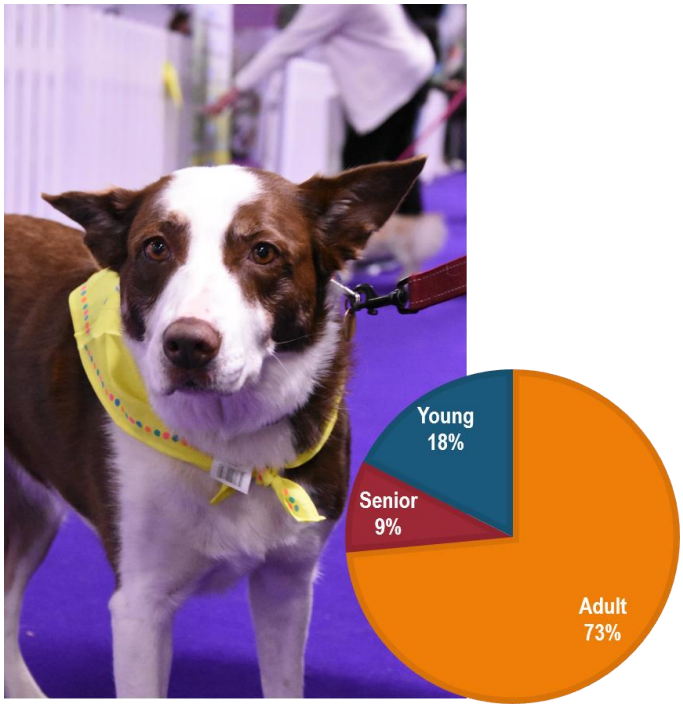

Figure 44 Dog 10: Senior female, ten years old mainly classified as an adult dog by assessors. Telomere Length shorter than expected for her age (Dog 10 relative telomere length: 0.68; Expected relative Telomere Length for the age: 0.74).

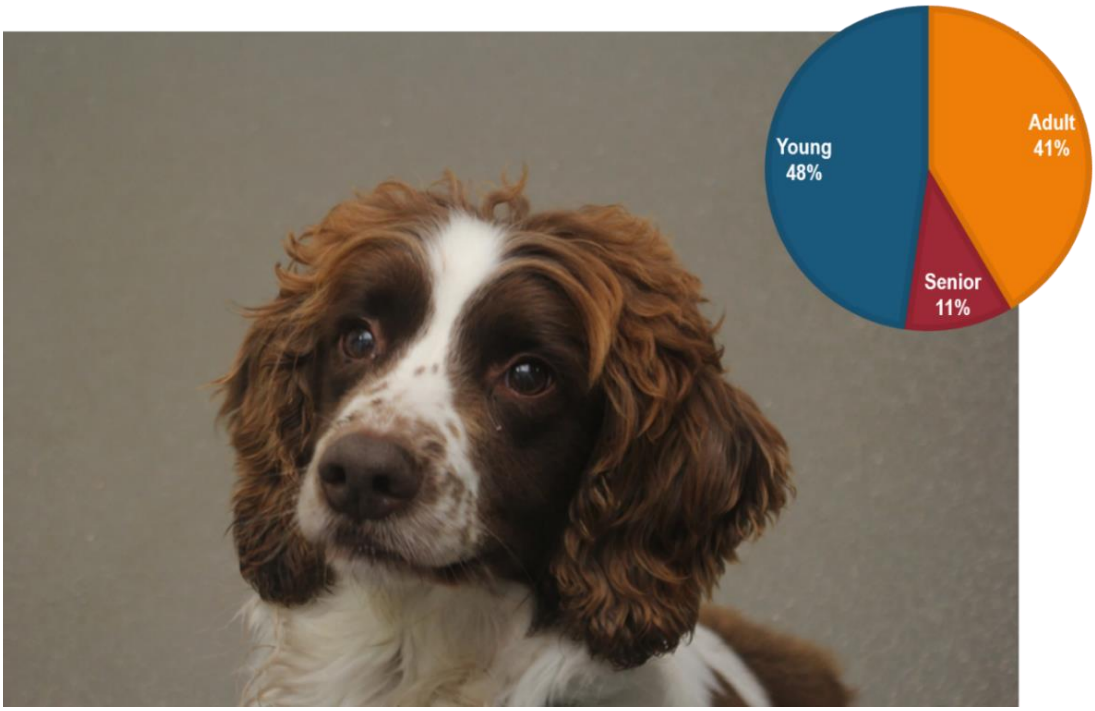

Figure 45 Dog 11: Senior male, twelve years old mainly classified as a young dog by assessors. Telomere length longer than expected for his age (Dog 10 relative Telomere Length: 0.75; Expected relative Telomere Length for the age: 0.67).

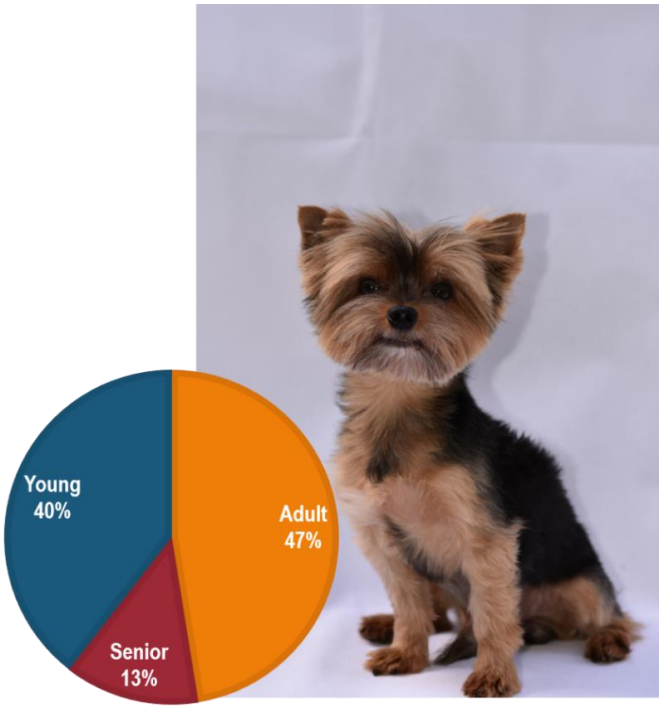

Figure 46 Dog 12 Senior male, seven years old mainly classified as an adult dog by assessors. Telomere length longer than expected for his age (Dog 12 relative Telomere Length: 0.78; Expected relative Telomere Length for the age: 0.74).

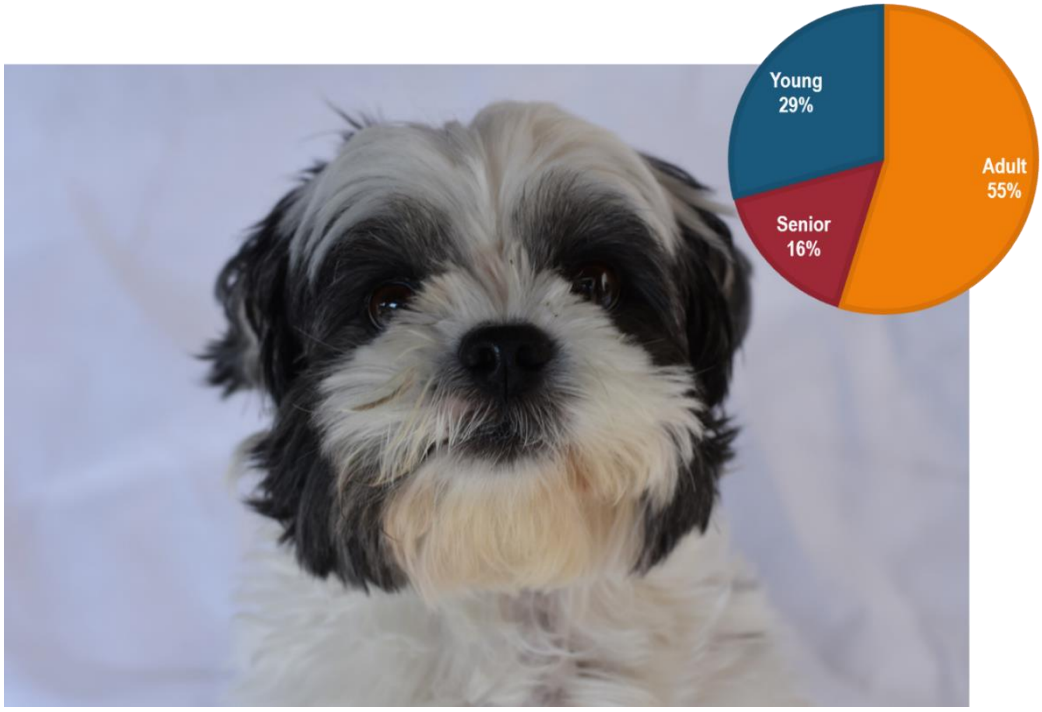

Figure 47 Dog 13: Senior male, eight years old mainly classified as an adult dog by assessors. Telomere length longer than expected for his age (Dog 13 relative Telomere Length: 0.90; Expected relative Telomere Length for the age: 0.80).

Figure 48 Dog 14: Senior female, six years old mainly classified as a young dog by assessors. Telomere length longer than expected for her age (Dog 13 relative Telomere Length: 0.81; Expected relative Telomere Length for the age: 0.75).

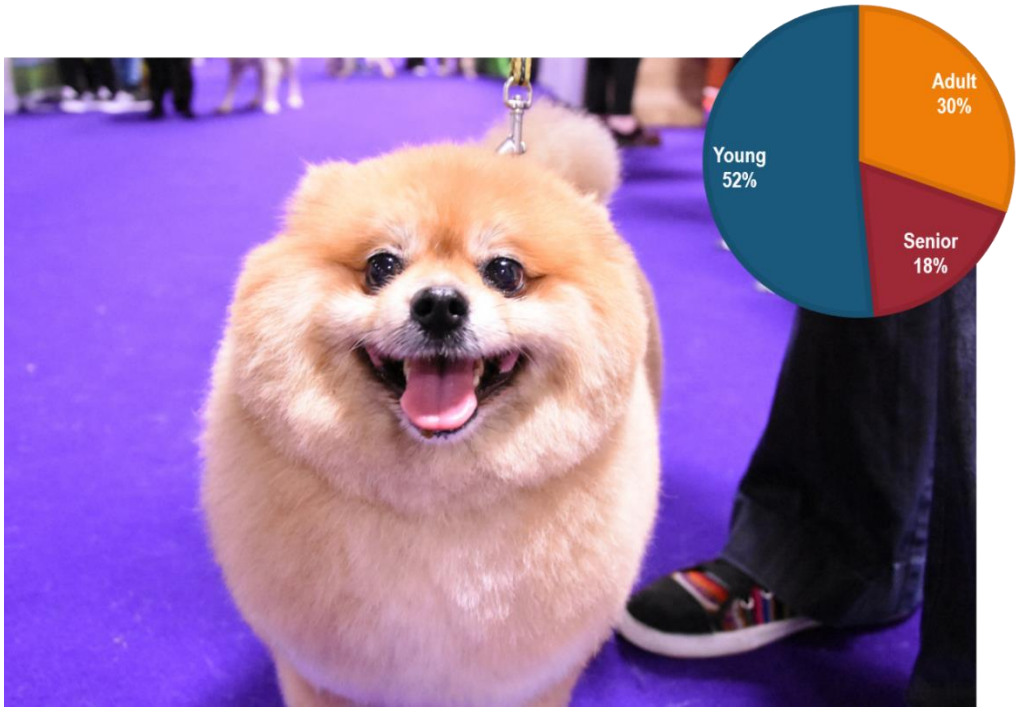

Figure 49 Dog 15: Senior male, seven years old mainly classified as a young dog by assessors. Telomere length shorter than expected for his age (Dog 14 relative Telomere Length: 0.67; Expected relative Telomere Length for the age: 0.74).

Figure 50 Dog 15: Young male, two years old mainly classified as an adult dog by assessors. Telomere length shorter than expected for his age (Dog 15 relative Telomere Length: 0.70; Expected relative Telomere Length for the age: 0.74).

Figure 51 Dog 16: Young male, two years old mainly classified as a senior dog by assessors. Telomere length shorter than expected for his age (Dog 16 relative Telomere Length: 0.64; Expected relative Telomere Length for the age: 0.74).

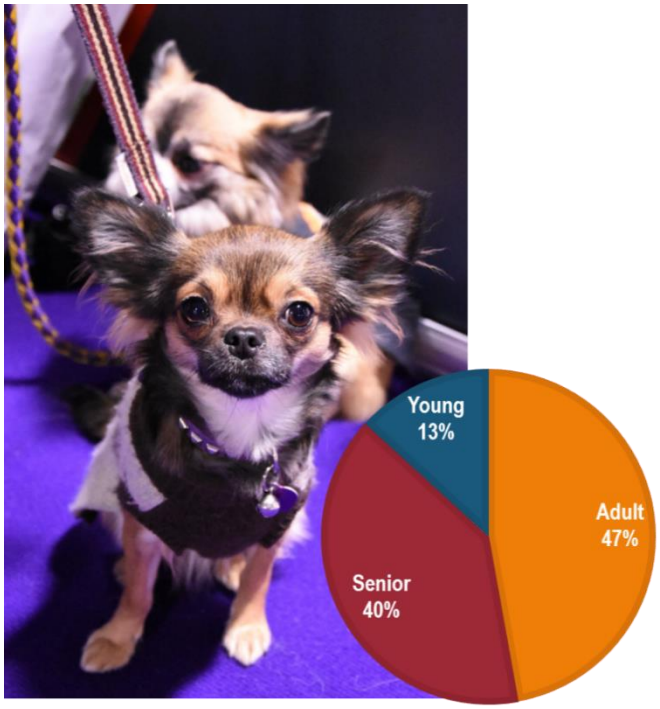

Figure 52 Dog 18: Young male, two years old mainly classified as an adult dog by assessors. Telomere length shorter than expected for his age (Dog 16 relative Telomere Length: 0.71; Expected relative Telomere Length for the age: 0.74).

6.6 Discussion

Although used for humans the concept of apparent age has not been used for assessing non-human species' health and welfare status (except for humans closest genetic relative the chimpanzee;(Kramer, 2012)), the present study shows that dogs' apparent age can be a tool to assess dogs' welfare. This promising result not only points that apparent age can be used in parallel with relative Telomere Length, but also highlights that people can identify young dogs that have the relative Telomere Length shorter than expect by their age, using photographs can be a more practical and accessible method to access quality of life in dogs than methods that involve physiological measurements (e.g. hormone levels).

Apparent age is a honest signal for individual ageing and individuals who look older than their chronological age develop the diseases of aging earlier (Hironobu Fukai, Hironori Takimoto, Yasue Mitsukura, & Minoru Fukumi, 2007). Usually, this evaluation is made through photos of the subjects and several characteristics such as hair colour and skin texture are analysed. People, either specialists or volunteers, were able to discern senior dogs from young dogs, hence, in further research, apparent age could be used by experienced dog handlers as a measure of biological age in other canids. For example, zoo keepers could use it to assess the apparent age of wolf species (using a blind experimental protocol).

The dogs that had the most accurate age predictions were senior dogs, most of them had the presence of grey hair in their muzzle, around the eyes and body, and displayed the development of cataracts; that is, well recognised signs of ageing (Bellows, 2015, King et al., 2016). Th young dogs that were identified with their chronological age correctly showed infant features described for puppies, such as large forehead, bright nose pigmentation and low-lying eyes (Kerswell, Butler, Bennett, & Hemsworth, 2010).

Dogs that had their age at least accurately predicted showed the aforementioned features in a paradoxical context where young dogs showed grey hair and senior dogs had ‘puppy-eyes’ and absence of grey hair. Dogs 17 and 18 were young dogs that had grey hair, the presence of grey hair usually is a sign of natural ageing, shown in humans, but also in dogs, however, premature greying can occur in young dogs that suffer from stress, fear or anxiety (King et al., 2016). Dogs 11 – 15 senior dogs of small or medium breeds were assigned as younger than they were, many

small breeds such as German Spitzes have retained the infant features into adulthood (Archer & Monton, 2011). These features then seem to be associated to biological age instead of chronological age, this would explain people's confusion in predicting correctly these dogs' ages.

Human facial photographs provide enough information for age estimation and relate positively with morbidity and mortality (Christensen et al., 2009; Gunn et al., 2009). There are studies showing people age at different rates and chronically stressed people not only have an older biological age, but also feel older relative to a given chronological age (Epel et al., 2004). Studies have proven the link between stress, increase of telomere attrition and risk factors for cardiovascular disease, poorer immune function in human; telomere attrition also relates to dogs' lifespan (Aydinonat et al., 2014; Fick et al., 2012; Gunn et al., 2013).

When specialists' and volunteers' scores for predicting dogs' ages were compared no significant difference was found. Studies indicate that the dogs' unique domestication process is the possible reason for the close relationship among humans and dogs, where dogs developed abilities to read human gestures and commands at the same time that humans can associate emotional states to different dog barking types (Pongrácz, Molnár, Dóka, & Miklósi, 2011; Pongrácz, Molnár, & Miklósi, 2006). Although expected that a more experienced person would predict more accurately a dogs' age having this close evolution process could be the underlying factor for people, regarding their experience, be able to age a dog from a photograph. However, due to the limitations of collecting data on Zooniverse we did not have access to demographic or canine expertise data from these participants. It is, therefore, possible

that our Zooniverse volunteers were all highly experienced dog owners, this may have been their motivation for participating in this study. Thus, the non-significant difference found may not be a true reflection of specialists versus non-specialists.

Humans can perceive a dog's age from photographs, either dogs that are ageing healthier than expected or dogs that are ageing prematurely. Animals that are biologically ageing more quickly than their chronological age are prone to a variety of diseases and reduced longevity, hence the importance of differentiating young from senior (Agrigoroaei et al., 2017). However, as showed in the previous chapter, telomeres could be repaired if adequate diet, exercise routine and social enrichment are offered to the dog, therefore the identification of young dogs ageing prematurely can offer a better ageing prospect for these dogs.

This is the first study to find relative Telomere Length associated to dogs' apparent age from an animal welfare perspective. Although our sample size was relatively small, the results are sufficiently strong to support the use of apparent age as a tool for assessing dogs' premature ageing. The use of dogs' apparent age should focus on identifying the outliers in a dog's group and using age categories makes this identification easier. Even very experienced specialists would encounter challenges in predicting the exact age of an individual in years. The essential action is to identify dogs that are ageing rapidly because it may be reversible. Future research in the animal welfare field should consider using apparent age and citizen science as a potential tool for quality of life assessment for both positive and negative welfare.

Chapter 7 Conclusions

7.1 General discussion

This research project has presented a general overview on dogs' welfare, contemplating new methodological and experimental approaches. Dogs have proven to be a great research model because within the species is found a great variety of backgrounds, lifestyles amongst other factors.

Currently it is discussed whether when evaluating animal welfare it is appropriate to use methods that will inflict pain for this reason it is advisable to develop and test non-invasive approaches (Swinbourne, Janssen, Phillips, & Johnston, 2014). Most studies are now using non-invasive methods such as collecting urine, faecal and saliva samples instead of collecting blood to evaluated physiological measures of animal welfare (Ash et al., 2018; Cook, 2012; Hulsman et al., 2011; Rupert Palme, 2005; Touma & Palme, 2005). Buccal telomere length is a non-invasive, possibly less expensive, method for examining telomere length than blood sampling, which would be invasive. Buccal swabs have also proven to be logistically a simpler collection method because samples occupy less physical space and can be kept under room temperature (i.e., no need for specialised freezers).

The commonest non-invasive method used to evaluate animal welfare from physiology is using faecal samples and several studies have been performed with different bird and mammals species (Cook, 2012; Nelson, Creel, & Cypher, 2015; Touma & Palme, 2005). However, the outcomes from faecal cortisol analysis provide information about a window of time, usually few a days or months, differently from relative Telomere Length, which information refers to a life time scale (Bateson,

2016; Rupert Palme, 2005). Faecal samples could give information over longer periods, however, due to logistics it would be harder to sample an individual daily or weekly over a period of years (Möstl & Palme, 2002b). Another factor to be consider is that cortisol studies are usually associated to poor (negative) welfare, where the cortisol levels in presence of the stimulus would be higher than the basal level. Nevertheless, if cortisol levels are at basal level this could be used to determine if the animal is in a positive or neutral welfare state. The importance of studying positive animal welfare has been highlighted, but the suggestions of physiological markers to evaluate positive states were: electroencephalography (EEG), positron emission tomography (PET) and functional magnetic resonance and imaging (fMRI), approaches that are invasive or impractical for many researchers (Yeates & Main, 2008).

Although several predicted associations in relative Telomere Length and lifestyle were not encountered in the present research, the results encountered strongly indicated that a dog's environment is related to variation in relative Telomere Length. Though telomeres and age are usually related with younger individuals presenting longer telomeres, the present results did not find this correlation, possibly due to the heterogenous population of dogs sampled and the consequential small sample size. However, there are studies showing that fast telomere shortening can occur during a young age and possibly juvenile stage may play an important role in determining telomere length in adult stage (Baerlocher, Rice, Vulto, & Lansdorp, 2007; Fairlie et al., 2016; Hall et al., 2004; Seeker, Ilska, Psifidi, Wilbourn, Underwood, Fairlie, Holland, Froy, Bagnall, et al., 2018). Dogs from large breeds were the ones that presented a longer telomere length mean contrary to what previous studies have shown (Fick et al., 2012). Telomere length is mainly inherited from parents and it increases with age

in sperm cells, which results in offspring conceived by older fathers having longer telomeres than the ones conceived by younger fathers (Olsson et al., 2011).

The animal welfare status of animals used in laboratory research is currently debated and although dogs in UK are classified as a specially protected species; this does not mean that their welfare state is assured in other countries (*Animals (Scientific Procedures) Act 1986*, 2012). Most studies investigating the welfare of dogs in a laboratory setting explore the physical and social enrichment, however, the assessment of welfare intervention results are usually through the observation of behaviours, such as the reduction of stereotypies or reduction of barking (Coppinger & Zuccotti, 1999; Timmins, Cliff, Day, Hart, Hart, Hubrecht, Hurley, Philips, et al., 2007; Wells, 2004). Present results indicated that relative Telomere Length can also be used to evaluate the positive effects of enrichment implementation in a laboratory environment. Because laboratory animals are susceptive to a chronic stress state which could result in reduced longevity this could be a promising approach to monitor their cumulative life experiences and to promote small husbandry changes that could have a major positive impact on an animal's quality of life.

7.2 Limitations

One of the major limitations of the present research was the sample size, although in total 290 dogs were sampled when taking into consideration their sex, age and background the numbers diminished leaving us with only one 14-year-old dog, for example. Despite this being a reality in the animal welfare field, it can prevent us from finding results that would represent an entire species or population.

In Chapter 3 due to logistics, faecal samples were only collected from dogs that were kept in kennels for this reason our sample size was small. No samples

from pet dogs, our biggest group sampled, were collected as owners did not wish to store faecal matter in their fridge. We attribute the moderate strength correlation between cortisol and telomere length due to small sample size. Studies investigating the association of human cortisol levels and telomere length either have a bigger sample size or sample a smaller group for a longer time period (Choi et al., 2008; Woody et al., 2017).

In Chapter 4 the two main limitations were sample size and lack of continuity, although we sampled as many dogs as it was possible there were not many senior dogs available. The impossibility to carry a longitudinal; study with all dogs were due three main reasons: (1) most pet dogs were sampled in an annual event and their participation in the event the following year could not be confirmed and privacy issues concerning contacting and identifying dog owners; (2) half of the sampling occurred in Brazil which impacted logistically on the possibility of re-sampling; (3) the timeframe of our experimental work was less than 3 years and more time would be needed for a longitudinal understanding of dogs' telomere dynamics. For these reasons we could not confirm that short telomeres lead to reduced longevity or increased disease in dogs.

In Chapter 5 we wanted to observe the biological change in telomere length over a year period, for this reason we did not plan any experiment, and the social enrichment was implemented by the institution responsible for the dogs (we decided on this approach as it became part of the dogs' husbandry rather than us requesting the animal care givers to undertake work for us). If we were able to conduct a planned (designed) experiment to evaluate the effect of social enrichment on dogs' relative Telomere Length we would have implemented both a control and an experimental

group (as Epel and collaborators (2016) did in their experiment when comparing telomere growth between two groups). Another point to be considered is that instead of sampling the same dogs only after a year, the sampling could have done before the enrichment be implemented, during the enrichment period and after a month from the last enrichment session. This sampling could provide more information regarding the rate of telomere growth and how long the growth effect lasts. The reason this was not done was due to the reluctance of the laboratory animal care staff to be involved in this sampling (in fact, we did all the sampling). We could not do frequent sampling due to the laboratory being in Brazil.

In Chapter 6 it is likely that small number of assessors impact the results. Although we collected personal information regarding sex, age and time working with dogs it was not possible to collect the same information about the volunteers. The Zooniverse platform is designed to assure the anonymity of the assessor, whereas this works from an ethical perspective it reduces the general understating of the project assessors. The dogs' pictures could have been assessed only by women, or by people who have own a dog for a long time or by biological researchers, however, we do not have any information that either confirms or refutes these hypotheses. Another technical limitation was that not all dogs that had their age assessed from photographs had their telomeres measured before. This was due to budget containments for laboratory procedures, as Pet dogs were the group that were most sampled when compared to other dog groups, we selected 84 dogs to have their DNA extracted and further analysis conducted.

7.3 Future research

This study found that different backgrounds had a significant impact on relative Telomere Length of dogs but because there were so many factors to be considered for each dog group it was difficult to affirm what factors were directly associated with their telomere length. As studies investigating factors that impact telomeres consider diet and exercise levels, we advise that future studies with dogs should also measure activity levels with accelerometers or activity budget dog collars (Arsenis et al., 2015). Nutrition is also a factor that could be taken into consideration, dog food that ranges from poor to premium quality can also play an important role in a dog's ageing just as nutrient rich diets play in human ageing (Boccardi et al., 2013).

This study discussed the dog's apparent age evaluated by volunteers, which can be a novel tool in animal welfare assessment. The citizen science approach connects science and education and because it can provide reliable data is considered to be a powerful approach that can solve the processing of large digital data sets while engaging non-traditional audience (Newman et al., 2012).

Experiments based on image recognition showed that computers can easily identify patterns in pictures, so dogs' apparent age could also be assessed by computers if an approach based on deep Convolutional Neural Networks is used (CNNs) (Medathati, Neumann, Masson, & Kornprobst, 2016). One advantage of using this method is the potential to increase the number of photographs that can be assessed, while 60 pictures assessment was considered long by people, in this study, a computer can easily analyse thousands of photographs in a short period of time (Fukai, Takimoto, Mitsukura, & Fukumi, 2007).

Future research can combine both citizen science and machine learning approaches if researchers design a website where owners could upload their dog's pictures and information and at the same time evaluate other dogs' apparent age pictures. The uploaded pictures could be assessed by computers using the CNNs analysis the results could be compared with the evaluations made by people. However, a large training data set for the CNN would be required (Oquab, Bottou, Laptev, & Sivic, 2014), this should not be a problem given the global population of domestic dogs and the frequency with which their owners like to photograph them.

In the final experimental chapter, we investigated the potential of a novel non-invasive approach to animal welfare evaluations. Apparent age is associated with mortality or age-related disease but only one study was conducted with a non-human species (and this was humans closest genetic relative, the chimpanzee) using this method to associates apparent age and health status (Sumner et al., 1989). Results showed that people not only can predict dogs' apparent age from photographs but were able to identify the outliers – dogs that are old, but look younger and dogs that are young, but look older than their chronological age. This result is potentially applicable in contexts such as dog shelters that receive dogs that might come from harsh environments. Being able to identify young dogs that are ageing rapidly can enable the shelter staff or the adopting family to reverse this effect implementing good welfare practices, good nutrition and activity.

7.4 Conclusion

In conclusion this work showed an approach that measures the overall cumulative life experiences of a dog's life because it incorporates both negative and positive experiences, whereas most animal welfare assessments contemplate only one

polarity. With apparent age instead of looking to a momentary situation we have a scale that can provide information either about good welfare, (when you look younger than you are) and bad welfare (when you look older than you are).

On a technical level both methodologies (apparent age and telomere attrition) work for dogs, buccal swabs can be used for dogs' telomere length investigations and dogs' telomere lengths are associated with cortisol levels, which adds more options to animal welfare assessment methods. From an experimental perspective we identified background factors that impact a dog's telomere length, we observed that a laboratory dog's telomere length increases over a year with the addition of social enrichment and people's assessment of dogs' apparent age might be related to a dog's cumulative life experience. No study with a non-human species has used multiple background factors to understand their role on telomere attrition dynamics as presented in this study.

The results of this research could have significant economic implications for pet owners and for institutions that have working dogs. Early detection of accelerated aging should mean that medical problems are detect at an early stage meaning that treatment is often simpler, less costly and with better outcomes (Stolz et al., 2009). For working dogs this might increase their working life whilst still maintain their welfare, given the cost of training a guide dog for the blind in the UK is £55,000.00 (Guide Dogs, 2017) even increasing working life by six months would significantly reduce costs.

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Appendix 1

Do you have a dog?
Are you curious to know how cumulative life-time experiences influences your dog's welfare?

My name is Luisa Dutra and I am a PhD researcher at the University of Salford and I would like to invite you and your dog to participate in my research project.

Animal welfare is assumed to be influenced by the cumulative effects of the positive and negative events experienced by an individual. Measuring stress is the main mechanism to evaluate welfare. Nowadays the methods used to assess stress are not available to everyone or are invasive, with that in mind a good measure of cumulative experience needs to be validated for animals. We believe that the way a dog looks can be related to his welfare and health. We aim to investigate if the used methods to assess stress are related with a dog's perceived age.

This research poses several questions. It will:

1. Test the different methods to assess dog stress.
2. Investigate how a dog's perceived age can be a predictor of their welfare.
3. Look at how changes in a dog's routine, such as play and walks, can reverse negative experiences effects.

I will be more than happy to provide you with more information regarding each question. You are encouraged to take part in all.

If you have any questions or require more information please do not hesitate to contact me.

Yours faithfully,

Luisa Dutra
PhD student
School of Environment and Life Sciences
University of Salford

Appendix 2

Participant Information Sheet

Study Title

Validating perceived age as a tool to evaluate canid wellbeing

Invitation Paragraph

I would like to invite your dog to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for your dog and yourself. Please take time to read the following information carefully.

What is the purpose of the study?

The aim of this project is to develop a simple and non-invasive system to evaluate the cumulative lifetime experience to validate age as a reliable tool to assess dog welfare.

Nowadays animal welfare is a widespread concern and assessing individuals' well-being is extensively discussed. Progress in improving animal welfare is currently limited by the lack of objective methods for assessing lifetime experience. Animal welfare is assumed to be influenced by the cumulative effects of the positive and negative events experienced by an individual. Measuring stress is the main mechanism to evaluate welfare. One way to assess animals´ life quality is measuring stress hormone levels because they are strongly associated with individual's health. Stressed individuals are more prone to develop cardiovascular diseases and have poorer immune responses. A molecular way to estimate life quality is measuring pieces of the animal's chromosomes called telomeres, once they reach a short length they induce cell death and thus, their length is associated with stress and life span. Looking older than your real (chronological) age is an indicator of a stressful life as you are aging

biologically faster than you should be. In humans looking old for your age, which can be assessed using facial photographs (perceived age) is associated with illness and death. A good measure of cumulative experience needs to be validated for non-human animals. The present study aims to investigate stress hormone levels, telomere lengths and the association of these factors with a dog's perceived age from a photograph.

Does my dog have to take part?

Taking part in the research is entirely voluntary. Prior to starting the trials I will describe the study to you. You will then be asked to sign a consent form to show you agree to your dog (and yourself) taking part. You are free to withdraw your dog at any time, without giving a reason.

What will happen to my dog and me if I take part?

The study will:

1. I will take a swab sample from your dog's cheek to check their stress level.
2. I will take a picture of your dog's face and body to evaluate their perceived age.

What are the possible disadvantages and risks of my dog taking part?

The only disadvantage is that some dogs don't like to have their mouth touched. To help with this issue we offer a positive training to desensitize your dog. Having a dog that is used to have its mouth touched facilitates owners to check dental health and administrating medicines. All the methods used in this study have been designed to

minimise discomfort and be time effective and convenient for you and your dog. There are no risks associated with taking part in the study.

What are the possible benefits of my dog taking part?

Your participation in the study will help us better understand new ways to measure stress in dogs.

Will my dog's and my taking part in the study be kept confidential?

All information, which is collected about you during the course of the research will be kept strictly confidential. No names or personally-identifiable information will be included in the thesis or any published results. The researcher will be the only person who has access to any identifiable data. Any paperwork that has been obtained, which contains any information about you, and all collected data will be securely filed in a lockable cabinet in the researcher's office and accessed only by the researcher. All electronic data will be protected by a password known only by the researcher. All data that has been collected shall be destroyed three years after the conclusion of the research.

What will happen if I and my dog don't carry on with the study?

If you withdraw your dog from the study we will destroy all your identifiable information and video recorded observations and/or trials, but we may use the data collected up to your dog's withdrawal. Your dog will also be made anonymous in the thesis or any published results.

What will happen to the results of the research study?

The results of the study will form part of the researcher's PhD thesis. The results will also be presented at conferences and may be published.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researcher who will do their best to answer your questions (contact details can be found below).

If you remain unhappy having spoken to the researcher, you should contact the researcher's supervisor:

Prof Robert Young

School of the Environment & Life Sciences

University of Salford

Email: r.j.young@salford.ac.uk

Tel: 0161-2952058

If you remain unhappy and wish to complain formally you can do this through the formal complaints procedure of the University of Salford. The student's supervisor can provide details of the complaints procedure if required.

Further information and contact details:

Specific information about this research project can be obtained at any time from the researcher:

Luisa Dutra

School of Environment & Life Sciences

University of Salford

Mobile: 07843848179

Email: l.m.l.dutra@edu.salford.ac.uk

Appendix 3

Ethical Approval Reference Number: STR1617-22

Name of Researcher: Luisa Dutra

I confirm that I have read and understood the information sheet for the above study (version 1: 01/02/16) and understand what my dog's, and my, contribution will be	Yes	No
I have been given the opportunity to ask questions (face to face, via telephone and/or e-mail)	Yes	No
I agree to have my dog photographed during the study.	Yes	No
I agree to have my dog's mouth swab sampled during the study.	Yes	No
I understand that my dog's, and my, participation is voluntary and that I can withdraw my dog from the research at any time without giving any reason.	Yes	No
I understand how the researcher will use my dog's samples, who will see them, and how the data will be stored.	Yes	No
I agree to my dog taking part in the above study	Yes	No

Name of participant……

Signature.....……...……

Date….....……....……

Name of researcher taking consent……

Appendix 4

25 January 2017

Luisa Dutra

Dear Luisa,

RE: ETHICS APPLICATION STR1617-22 – Validating perceived age as a tool to evaluate canids' wellbeing

Based on the information you provided, I am pleased to inform you that your application STR1617-22 has been approved.

If there are any changes to the project and/ or its methodology, please inform the Panel as soon as possible by contacting S&T-ResearchEthics@salford.ac.uk

Yours sincerely,

Prof Mohammed Arif

Chair of the Science & Technology Research Ethics Panel Professor of Sustainability and Process Management School of Built Environment

University of Salford

Maxwell Building, The Crescent Greater Manchester, UK M5 4WT Phone: + 44 161 295 6829

Email: m.arif@salford.ac.uk

www.salford.ac.uk/ethics

Appendix 5

Authorisation No: ITIMP16.1096

DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

AUTHORISATION FOR THE IMPORTATION FROM THIRD COUNTRIES OF RESEARCH AND DIAGNOSTIC SAMPLES

European Communities Act 1972

TRADE IN ANIMALS AND RELATED PRODUCTS REGULATIONS 2011
ANIMAL BY-PRODUCTS (ENFORCEMENT) (ENGLAND) REGULATIONS 2013,
ANIMAL BY-PRODUCTS (ENFORCEMENT) (SCOTLAND) REGULATIONS 2013,
ANIMAL BY- PRODUCTS (ENFORCEMENT) (WALES) REGULATIONS 2014

The Secretary of State for Environment, Food and Rural Affairs, by this authorisatio n issued under the terms of Paragraph 4 of Schedule 3 of the Trade in Animal and Related Products Regulations 2011 authorises:

c/o Dr Robert John Young University of Salford School of Environment and Life Sciences Peel Building, Room G21 M5 4WT	Name and full postal address of importer responsible for consignment
	Name and full postal address of destination premises (if different from importer)

Subject to and in accordance with the conditions set out below, the landing in England of:

Dog faecal, buccal swabs and blood samples, intended for particular studies or analyses only. (Not for resale).	Product
Brazil	Cu.ntries of - origin
All ports and airports in England	Ports of entry
24/1 1/2018	Expiry Date

Dated: 24/;1;1/2016

Officer of the Department for Environment, Food and Rural Affairs

1

Conditions attached to this authorisation

1. This authorisation is valid for multiple consignments and the net weight per consignment must not exceed 1 kg.

2. The material must be packed in leak-proof sealed containers.

3. All inner and outer packaging must be swabbed with suitable disinfectant before leaving the exporting address.

4. Irrespective of the mode of transport, all specimens must be packaged so that they fully comply with the requirements of relevant Post Office or International Air Transport Association (IATA) regulations.

5. The packaging must be clearly labelled to indicate the nature of the product, that this is intended for *in vitro* use for research *and that it is not for human or animal consumption.*

6. Each consignment must be accompanied by a copy of this import authorisation and a commercial document which must confirm:

   i) The description of the material and the animal species of origin;

   ii) The category of the material as defined in Articles 8, 9 or 10 of Regulation (EC) No 1069/2009[1];

   iii) The quantity of the material;

   iv) The place of origin and the place of despatch of the material;

   v) The name and the address of the consignor;

   vi) The name and address of the consignee and/or user;

7. Each consignment must be accompanied by a declaration (see note D) on headed/official paper confirming that:

   i) That the products are **not** derived from animals known or suspected to be infected with a pathogen which causes a notifiable disease to which the animals from which the products are derived are susceptible according to European Regulations• or the Animal Health Regulations of the exporting country; and
   ii) That the products do not originate from animals in a premises or region or zone of a country that is subject to official restrictions due to a notifiable disease to which the animals are susceptible according to European or other National Animal Health Regulations.

   •council Directive 82/894/EEC of 21 December 1982 (as amended) on the notification of **animal diseases within the Community**

8. **In accordance with Article 27.2 of Regulation (EU) 142/2011, research and diagnostic samples from Third countries which are intended to be imported via a Member State other than the MS of destination must come in at an approved Border Inspection Post (BIP). They will not be subject to veterinary checks but the BIP must inform the MS of destination of the introduction of the sample by means of the TRACES system (https://webgate.ec.europa.eu/sanco/traces/)**

[1] OJ No **L** 300, 14.11.2009, p.1.
•council Directive 82/894/EEC of 21 December 1982 (as amended) on the notification of animal diseases within the Community

9. The consignment must be sent directly from the point of entry into the Union to the authorised user at the destination address on page 1.

10. The transporter and destination address must be registered or approved (see note F) under the Animal By-Products (Enforcement) (England) Regulations 2013 (ABPE) before commencing operations.

11. The products must remain in their original wrapping at all times until their arrival at the destination address on page 1.

12. Users shall take all necessary measures to avoid the spreading of diseases communicable to humans or animals during the handling of the materials under their control, in particular by way of the application of good laboratory practice.

13. The samples and material derived from the samples shall be for *in vitro* use only.

14. Samples to be handled and stored under containment level 2 conditions

15. None of the material to which this authorisation relates shall be used for human consumption under any circumstances.

16. Any subsequent use of these products for purposes other than those referred to in point 38 of annex 1 of Regulation (EU) No 142/2011, shall be prohibited.

17. Importers shall keep a register of consignments of samples imported under this authorisation, which should contain the information referred to in condition 6 above as well as the date and method of disposal.

18. Unless they are kept for reference purposes, or re-dispatched to the third country of origin, research and diagnostic samples and any products derived from their use, shall be disposed of:

    I) As waste by incineration or co-incineration;

    ii) By pressure sterilisation and subsequent disposal or use in accordance with Articles 12, 13 and 14 of Regulation (EC) No 1069/2009.

    iii) In accordance with point 4(b) of Section 1 of Chapter I of Annex VI of Regulation (EU) No 142/2011 in cases of:

    (a) Quantities not exceeding 2000 ml; and

    (b) Provided the samples or derived products have been produced and dispatched from third countries or parts of third countries, from which Member States authorise imports of fresh meat of domestic bovine animals, which are listed in Part I of Annex II to Regulation (EU) No 206/2010.

19. Any breach of these conditions must be reported to the local Animal and Plant Health Agency (APHA) Office.

NOTES

A. When expired or exhausted this authorisation is to be returned to the address below.

B. Please note that while this authorisation was current at the time of its issue, conditions can be subject to frequent change and importers are advised to check the latest position with Animal and Plant Health Agency, Imports Team, Carlisle, at the address below.

C. It is the responsibility of the importer to follow good laboratory practice standards and to prevent the sample entering the environment in any manner. The material must be produced, processed, transported, handled, labelled, stored, used and disposed of in accordance with the Animal By-products Regulations.

D. All declarations must be written on headed paper, dated and signed.

E. In accordance with Annex VIII, Chapter III, point 5 of Regulation (EU) No 142/2011, all records and related documentation associated with material imported under this authorisation must be kept for a minimum of 24 months for presentation to the competent authority.

F. For information on registration/approval, please see the website:
https://www.gov.uk/animal-by-product-categories-site-approval-hygiene-and-disposal#getting-your-site-approved-or-registered

CAUTION

It is the importer's responsibility to ensure that any import covered by this authorisation complies with the terms and conditions as set out. If you cannot comply with any of the conditions above, please contact the APHA Imports Team.

Any breach of any conditions attached to this Authorisation will constitute an offence against regulation 39 of the Trade in Animals and Related Products Regulations 2011 or regulation 17 of the Animal By-products (Enforcement) (England) Regulations 2013.

Any samples imported under this authorisation are only for use at the destination premises on page 1. If you wish to move these samples to another premises for any purpose other than destruction, please contact the APHA Imports Team.

CONTACT FOR FURTHER INFORMATION

Animal and Plant Health Agency
Imports Team
Centre for International Trade – Carlisle
Eden Bridge House,
Lowther Street,
Carlisle,
CA3 8DX
t 03000 200 301
e: imports@apha.gsi.gov.uk

Appendix 5

Blood and Swab DNA Extraction using DNeasy® Blood & Tissue Kit – QIAGEN

Preparation of the sample

- Set the incubator or water bath to **56ºC** and **94ºC**.
- Swabs: detach the cotton head from the plastic cane and place it in a microcentrifuge tube.
- Dried Blood Spot from FTA® card: cut all the surface containing blood and place it in a microcentrifuge tube.

Lyse of the sample

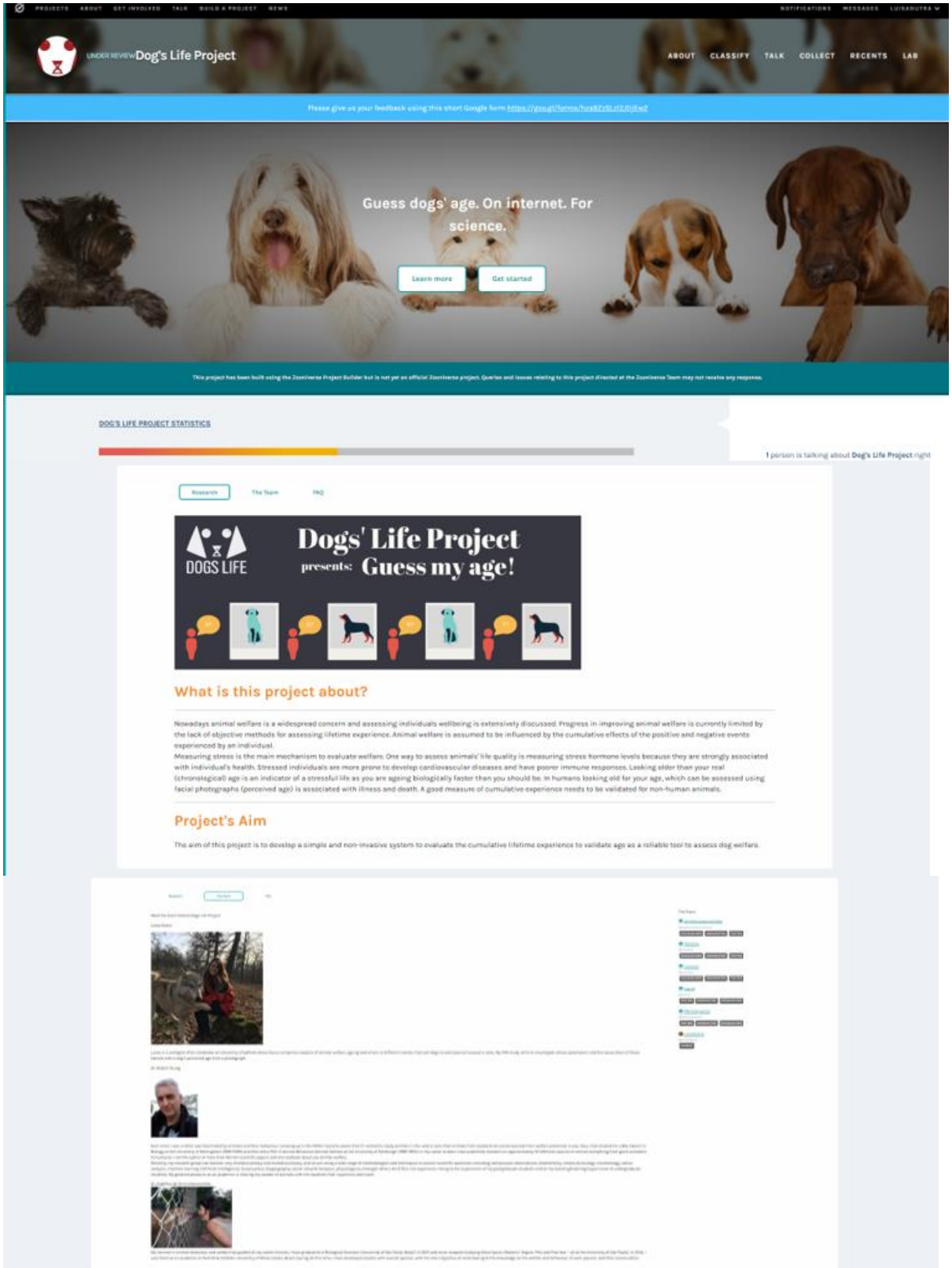
- Add **180 µL Buffer ATL** and vortex to mix. Place the sample in a water bath for **10 min at 94ºC**. Let the sample cool down. Add **20 µL Proteinase K Solution**. Spin the sample briefly, then vortex it.
- Incubate at **56ºC** until complete lysis for **1:30 hours**. Vortex occasionally.

DNA extraction

- After digestion, centrifuge at **2.000 rpm** for **15 seconds**.
- Vortex the samples for 30s, add **200 µL Buffer AL,** vortex to mix. Then add **200 µL ethanol** (100%) and mix again carefully by vortexing.
- Pipet each sample into a column placed in a collection a tube, discard the left FTA® card and swab. Centrifuge for **1 min at 8000 rpm**. Discard the flow-through and collection tube.
- 1st wash: Add **500 µL Buffer AW1**. Centrifuge for **1 min at 8000 rpm**. Discard flow-through and place the column back into a new collection tube.
- 2nd wash: Add **500 µL Buffer AW2**. Centrifuge for a 3 min at 14.000 rpm. Discard flow-through and place the column back into a new collection tube.
- Transfer carefully the spin column to a new 1.5 ml microcentrifuge tube.
- Elute the DNA by adding **100 µL Buffer AE**. Incubate at room temperature for **2 minutes**. Centrifuge **1 min at 8000 rpm**.
- Store at **-20 ºC**

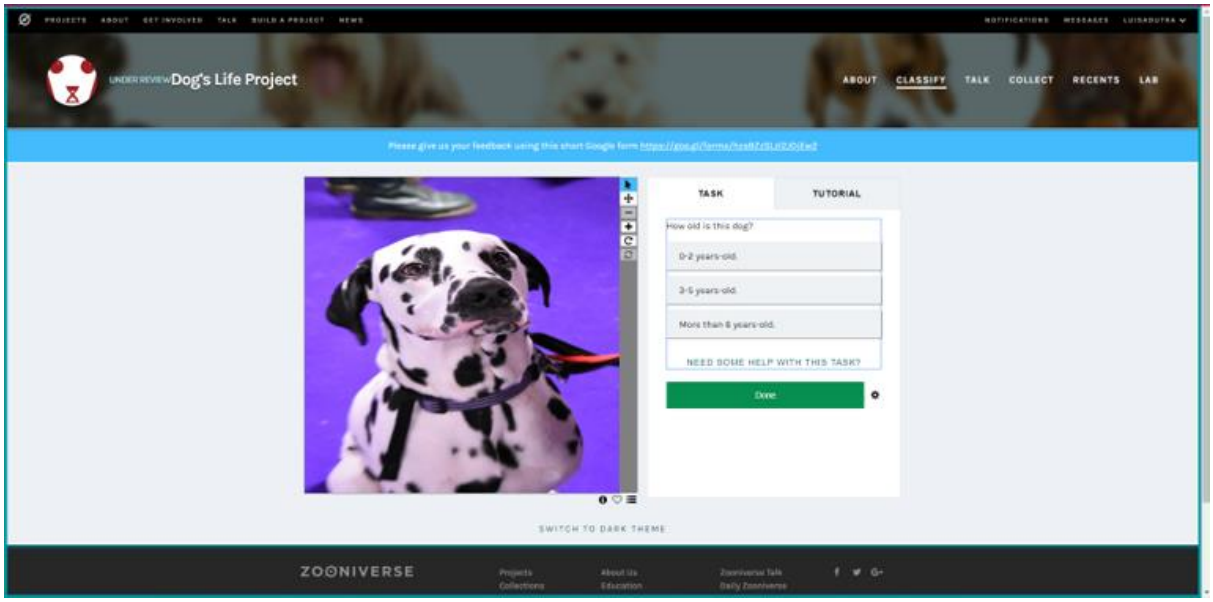
Appendix 6

Zooniverse Main Page, Project Presentation and Team.



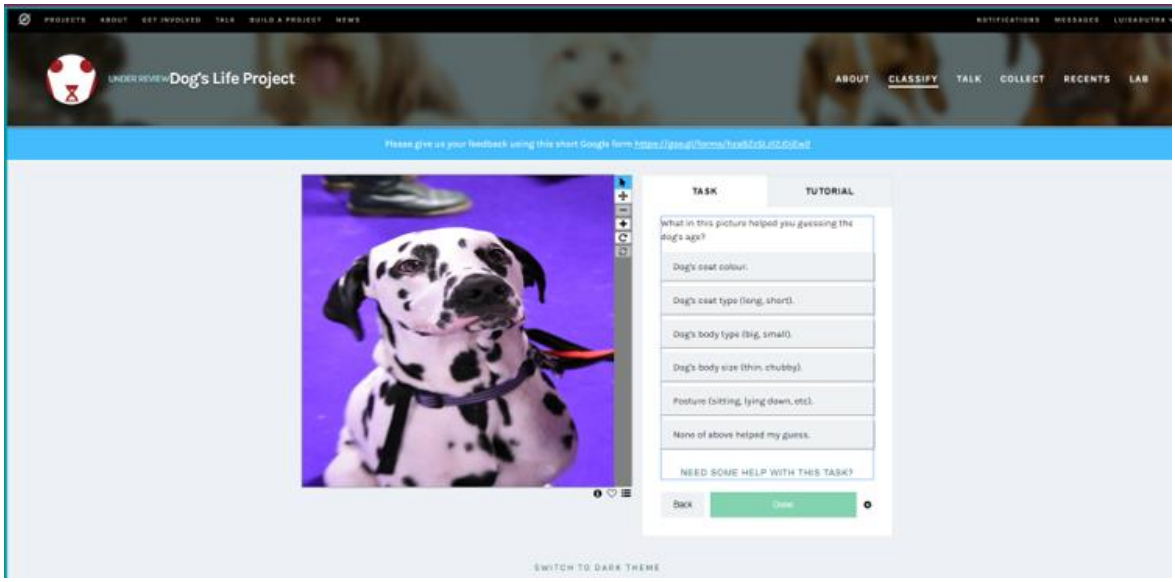
Appendix 7

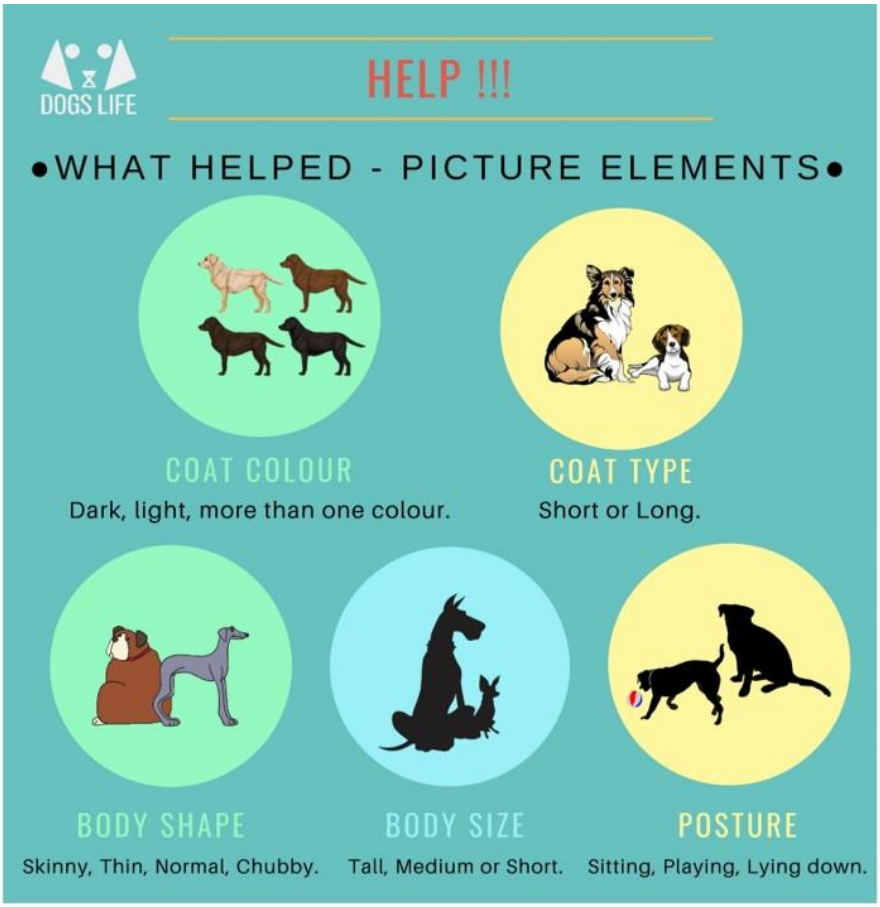
“Task 1: How old is this dog?” and Task 1 Help



Appendix 8

“Task 2: What in this picture helped you guessing the dogs’ age?” and Task 2 Help





Appendix 9

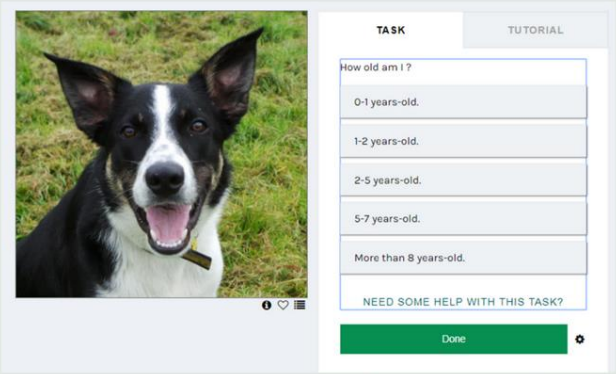
Dogs' Life Tutorial

YOUR HELP

We are using two ways to access dogs perceived age: people and computers.

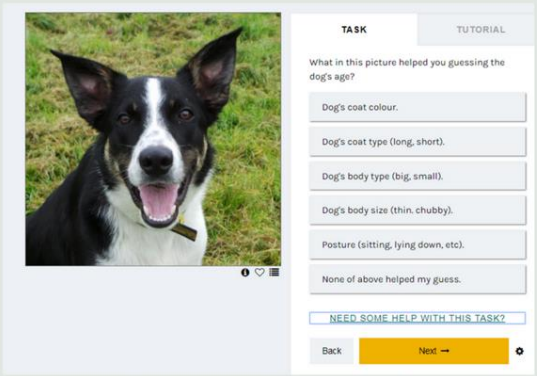
We want to understand how similiar or different are people and computer's guesses.

TASK 1



Here you need to guess how old is the dog.
You need to choose the best option and then click Done.
Having trouble? Click on Need some help.

TAKS 2



Here you can tell us what helped your guess. You can choose as many as you want, or None and then click on Next to the last step. Having trouble? Click on Need some help.

THANK YOU

You just helped science test different methods to assess ageing and stress in dogs.

Appendix 10

Dogs' correct predictions score, their true age and relative telomere length

Sample	ID	Correct predictions (%)	Category	Age	Telomere	Expected Telomere	CI (95%)
50	SS45_M_2	1.16	Young	2	0.712	0.750	(0.729, 0.770)
5	FPS117_F_10	8.14	Senior	10	0.683	0.745	(0.658, 0.829)
31	MPS12_M_12	10.47	Senior	12	0.751	0.674	(0.442, 0.904)
42	PS1_ M_7	13.95	Senior	7	0.776	0.742	(0.710, 0.774)
44	PS16_M_8	16.28	Senior	8	0.902	0.801	(0.660, 0.939)
53	WKDS12_F_5	18.60	Adult	5	0.81	0.752	(0.720, 0.783)
27	FPS89_M_1	23.26	Young	1	0.640	0.746	(0.722, 0.771)
36	PBS10_M_2	25.58	Young	2	0.806	0.750	(0.729, 0.770)
3	FPS113_M_7	27.91	Senior	7	0.670	0.742	(0.710, 0.774)
26	FPS87_M_4	30.23	Adult	4	0.782	0.727	(0.697, 0.756)
40	PBS34_M_6	30.23	Senior	6	0.844	0.751	(0.7140, 0.7870)
38	PBS24_M_1	31.40	Young	1	0.874	0.746	(0.722, 0.771)
25	FPS8_F_1	32.56	Young	1	0.617	0.746	(0.722, 0.771)
58	WS12_F_1	32.56	Young	1	0.676	0.746	(0.722, 0.771)
54	WKDS18_M_1	33.72	Young	1	0.715	0.746	(0.722, 0.771)
60	WS14_M_1	37.21	Young	1	0.697	0.746	(0.722, 0.771)
51	SS47_F_5	39.53	Adult	5	0.734	0.752	(0.722, 0.771)
4	FPS115_M_1	43.02	Young	1	0.689	0.746	(0.722, 0.771)
39	PBS26_M_4	44.19	Adult	4	0.723	0.727	(0.697, 0.756)
49	SS25_F_5	44.19	Adult	5	0.679	0.752	(0.720, 0.783)
29	MPS1_M_1	45.35	Young	1	0.839	0.746	(0.722, 0.771)
32	MPS14_M_7	45.35	Senior	7	0.768	0.742	(0.710, 0.774)

Sample	ID	Correct predictions (%)	Category	Age	Telomere	Expected Telomere	CI (95%)
46	PS7_F_5	45.35	Adult	5	0.706	0.752	(0.720, 0.783)
55	WKDS19_F_2	45.35	Young	2	0.725	0.750	(0.729, 0.770)
47	PS8_F_5	46.51	Adult	5	0.614	0.752	(0.720, 0.783)
59	WS13_M_3	46.51	Adult	3	0.693	0.762	(0.735, 0.788)
57	WKDS9_F_2	50.00	Young	2	0.846	0.750	(0.729, 0.770)
2	FPS106_F_02	51.16	Young	2	0.637	0.750	(0.729, 0.770)
35	MPS19_F_3	51.16	Adult	3	0.729	0.762	(0.735, 0.788)
56	WKDS24_F_5	52.33	Adult	5	0.682	0.752	(0.720, 0.783)
33	MPS15_M_14	53.49	Senior	14	0.757	0.76	*
34	MPS17_M_3	53.49	Adult	3	0.757	0.762	(0.735, 0.788)
37	PBS16_M_5	54.65	Adult	5	0.730	0.752	(0.720, 0.783)
7	FPS16_M_3	55.81	Adult	3	0.777	0.762	(0.735, 0.788)
43	PS13_M_2	55.81	Young	2	0.669	0.750	(0.729, 0.770)
1	FPS101_F_7	56.98	Senior	7	0.736	0.742	(0.710, 0.774)
52	SS5_M_2	59.30	Young	2	0.919	0.750	(0.729, 0.770)
16	FPS4_F_9	62.79	Senior	9	0.724	0.752	(0.695, 0.804)
45	PS18_F_13	74.42	Senior	13	0.646	0.792	(-1.047, 2.637)
6	FPS15_F_2	75.58	Young	2	0.744	0.750	(0.722, 0.771)
24	FPS73_M_7	80.23	Senior	7	0.747	0.742	(0.710, 0.774)
30	MPS11_M_ 12	81.40	Senior	12	0.570	0.674	(0.442, 0.904)
41	PBS5_F_6	95.35	Senior	6	0.820	0.751	(0.714, 0.787)
48	SS17_M_10	97.67	Senior	10	0.792	0.745	(0.658, 0.829)